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BIOLOGICAL ABSTRACTS
UNIVERSITY OF PENNSYLVANIA
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Studies on the Localization of Cholinesterase in Various Types of Muscle

T. KOVÁCS, A. KÖVÉR AND G. BALOGH
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Varga et al. ('54) have shown that myosin, the contractile protein of muscles, has cholinesterase activity and that this activity is the property of L-meromyosin, previously believed to be inactive enzymatically. It has also been shown (Varga et al., '57a,b) that when the otherwise tetanic gastrocnemius muscle, either in the early phase of ontogenesis, or in the course of dedifferentiation following motor denervation, shows tonic character, the activity of myosincholinesterase computed for 1 gm of muscle significantly increases. In a recent paper Kövér and Kovács ('57c) have reported that tonic muscles showing high acetylcholine sensitivity have much higher myosin cholinesterase activity than tetanic muscles in some species of the phylogenetic series, These observations suggest that the cholinesterase activity of muscle is not ascribable to the activity of acetylcholinesterase concentrating at the end plates alone, but involves in an about equal measure the activity of myosincholinesterase, distributed diffusely in muscle fibers and forming part of the contractile structure. In spite of the fact that muscle fibers themselves can be shown to possess considerable cholinesterase activity, most authors investigating the localization of cholinesterase in skeletal muscles focussed attention almost exclusively on the high acetylcholinesterase activity and content of the end plates. Portugalov ('55) was the first to point out that in certain cases cholinesterase activity could be demonstrated by histochemical methods along the entire length of the muscle fiber; moreover, on grounds of his observations concerning various muscles he has called attention to the circumstance that it is just in the so-called tonic muscles that the intracellular cholinesterase activity of muscle fibers is high. More recently Becket and Bourne ('57) have shown that the intracellular cholinesterase activity is demonstrable also in human muscles and that it is particularly increased in atrophied muscles. Our results and the observations mentioned tend to indicate that Portugalov and Becket and Bourne have demonstrated by histochemical methods just an increase of myosincholinesterase activity in different muscles. On grounds of this assumption we have investigated the localization of cholinesterase in muscles showing different activities; also, we have undertaken to determine whether the histochemically demonstrable intracellular cholinesterase activity of muscle fibers would change parallel with the changes in enzyme activity demonstrated *in vitro*.

METHODS

Kövér and Kovács ('57b) have reported that myosincholinesterase would split the acetylthiocholine used with the histochemical method of Koelle. Starting out from this observation, we have employed the method of Koelle and Friedenwald ('49). This method is suitable for use first of all for the determination of the location of high concentrations or highly active enzymes and thus cannot be employed in its original form in every case. Considering that myosincholinesterase can be found intracellularly and is distributed diffusely corresponding to the contractile structure and that its specific activity is much lower than that of acetylcholinesterase concentrating at the end plates, the method had to be modified, partly to create conditions optimal for enzyme function and partly to make it easier for acetylthiocholine to enter the intracellular space.

The method we have adopted may be outlined in brief as follows. Fixation is essential to preserve structure and to prevent diffusion of proteins. The common histological methods usually diminish the activity of the more sensitive enzymes. Koelle had placed frozen sections without previous fixation into an incubation mixture containing Na_2SO_4 in an end concentration of 24%, and thus prevented changes in structure by causing a precipitation of tissue proteins (Koelle, '51). He claimed that the use of Na_2SO_4 reduced the formation of "diffusion artifacts." We showed in *in vitro* experiments that even at concentrations substantially lower than that used by Koelle, Na_2SO_4 greatly diminished the activity of myosincholinesterase; therefore we did not use Na_2SO_4. Taxi ('52) reported that fixation with 10% formaldehyde at 18°C for three hours reduced by about 35% the cholinesterase activity of pancreas homogenates. At the same time, they called attention to the fact that the degree of cholinesterase inactivation is greatly dependent upon the concentration and temperature of the solution used. Taking these data into account, we allowed the specimens of tissue excised to stand in 8% neutralized formaldehyde at 0°C and found that in such conditions fixation caused only a small loss of cholinesterase activity.

To facilitate diffusion of quaternary ammonium salts, i.e., to enhance the permeability of the membrane, Portugalov ('55) recommended pretreatment with acetone at − 18°C for three hours. In his experiments intracellular activity proved to be much higher following such pretreatment. For example, by Koelle's method the unmyelinated nerve fibers have no demonstrable cholinesterase activity, although biochemical evidence suggests the presence of considerable cholinesterase activity, whereas Portugalov observed significant histochemical reaction or cholinesterase activity in the structure of the sheath of Schwann by his histochemical method involving pretreatment with acetone. Considering that our method and the pretreatment with acetone suggested by Portugalov yielded the same results, we assume that fixation with formaldehyde increases the permeability of the membrane for the quaternary homologues.

After fixation, we made frozen sections, 20 to 30 μ thick each, spread them over the surface of neutralized distilled water (+ 4°C) and washed out the formaldehyde by exchanging the distilled water several times over a period of half an hour. Subsequently the sections were placed into an incubation mixture (to be described in detail later) and were allowed to stand at 37°C for one hour in an incubator.

At first, Koelle had used incubation mixtures of higher pH, but found that such mixtures interfered with the formation of cuprithiocholine and thus with the color reaction; for this reason he has been using lower pH's recently. As the pH optimum of cholinesterase is around pH 8.0, including myosincholinesterase (Kövér, Kovács and Kőnig, '57) we thought it desirable to use incubation mixtures of optimum pH, with special reference to the low activity of myosin. As by using the method as originally described by Koelle we had found the development of the color reaction less satisfactory with using incubation mixtures of pH 8.0, we modified the method in the following way. The sections taken out from the incubation mixture were washed, after arresting enzyme activity and before development of the color reaction, with several changes of distilled water previously saturated with cuprithiocholine.

On grounds of the considerations outlined above we excluded Na_2SO_4 from the incubation mixture. We think it extremely important that the incubation mixture be saturated with cuprithiocholine, so as to prevent diffusion of the precipitate. Unlike the description of Koelle, we employed no bivalent ions in the incubation mixtures, because according to our data (Kövér, Kovács and Kőnig, '57) bivalent ions do not activate myosincholinesterase, though they activate acetylcholinesterase. Of course, in our experiments we strove to create optimum conditions for myosin, in the first place.

The reaction mixture we used had the following composition:

1. Copper glycine solution, 1 ml. (Glycine, 3.75 gm; $CuSO_4 \cdot 5\ H_2O$, 2.5 gm volume adjusted to 100 ml.)
2. Veronal-Na-HCl buffer, 0.05 M, pH 8.0, 3 ml.

3. Acetylthiocholine iodide solution, 1.2
. (Acetylthiocholine iodide (light),
mg; H_2O, 1.2 ml; 0.1 M $CuSO_4$, 0.4 ml;
xed, centrifuged and using the super-
tant.)
4. Cuprithiocholine, in traces.
5. Distilled water, 9.4 ml.

The sections were allowed to stand in
cubation mixtures for one hour in an
cubator at 37°C. After arresting en-
me activity, they were placed (with the
cubation vessels) into an incubator to
and therein at 60°C for 10 minutes.
n minutes later the sections taken out
om the incubator were washed with the
utralized, cuprithiocholine-saturated dis-
led water as described above, then the
lor reaction was developed with Na_2S
turated with copper sulfide. After that
e sections were washed with distilled
ater saturated with CuS. Subsequently
e used the preservation procedures em-
oyed in histology (dehydration, cover-
g by Canada balsam).

To inhibit cholinesterase activity 10^{-5} M
ysostigmine was used. As our primary
m was to study the activity of myosin-
olinesterase, some of the frozen sections
t without fixation from various species
ere extracted with KCl of low ionic
rength (0.02 μ) and KCl of high ionic
rength (0.6 μ) at 0°C, for from 60 to
0 minutes.

RESULTS

The localization of cholinesterase has
en studied by the above modified histo-
emical method in gastrocnemius mus-
es, normal ones from rabbits of various
es and denervated ones from adult rab-
ts. The first three pictures in figure 1
ow preparations made from rabbit mus-
es at different ages (A, two-day; B, two-
onth; C, 6-month rabbits). In each
cture the histochemical reaction of ace-
lcholinesterase concentrated at the end
ates is clearly visible, while in the fibers
gnificant activity can be seen in sections
and B only. The activity visible in sec-
on C is much lower, as it could be antici-
ated on grounds of our biochemical
xperiments. If we compare the cholines-
rase reactions of the muscle fibers from
nimals of different ages, it will be seen
at the intensity of the color reaction de-
reases with the advance of ontogenetical
development. Meanwhile, the structure of the end plates become more and more differentiated. This observation is in harmony with the data published by Varga, Kövér, Kovács and Hetényi ('57b), according to which in the early phase of ontogenetical development the specific activity of myosincholinesterase is high, then it decreases with the advance of time, becoming set at a constant level in adult animals. These data, in agreement with evidence reported by other workers, also indicate that in these muscles the amount of myosin is continually increasing (Hermann and Nicholas, '48; Robinson, '52a, b). Thus, the intensity of the histochemical reaction depends on these two factors, notably on the specific activity as well as the quantity of myosin.

Pictures D and E in figure 1 show the cholinesterase activity of the gastrocnemius muscle from an adult rabbit, 30 days after cutting the sciatic nerve. Comparing pictures D and E with picture C, it will be observable that unlike in normal muscles, in denervated ones cholinesterase activity is marked not only at the end plates, but inside the muscle fibers as well. Picture E is particularly noteworthy, as it shows areas rather distant from the end plates and yet the fibers show intense color reaction. We think that this color reaction cannot be explained in any way by the formation of some diffusion artifact.

Finally, picture F in figure 1 shows a preparation made from denervated muscle pre-treated with physostigmine. In this preparation no color reaction is visible either at the end plate or in the muscle fibers, owing to the inhibition of cholinesterase.

In figure 2 pictures made by the same technique can be seen, with the same magnification. Picture A shows the longitudinal muscles of earthworm (*Lumbricus terrestris*), B shows the adductor muscle of clam (*Anadonta cygnea*) and C shows the foot muscle of the same species. Picture C represents the appearance of the sole muscle of snail (*Helix pomatia*) and E. the back muscle of leech (*Hirudo medicinalis*). In each of them color reaction indicative of cholinesterase activity is visible in the area of muscle fibers. The intensity of the color reaction allows one

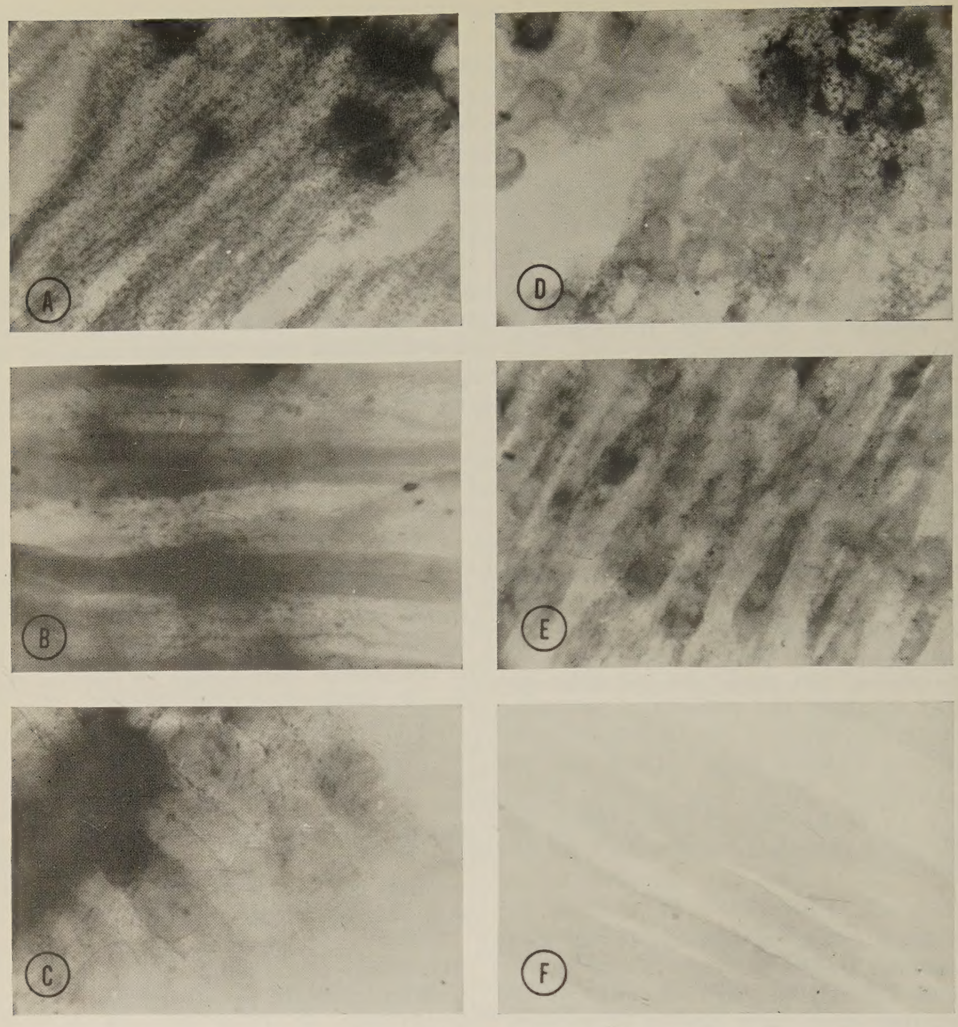

Fig. 1 Changes of the intracellular cholinesterase activity in gastrocnemius muscle of the rabbit, in the course of ontogenesis or following denervation; 30-μ sections. Incubation for one hour. A, two-day rabbit; B, two-month rabbit; C, 6-month rabbit. Sections from gastrocnemius muscle D and E. Sections of gastrocnemius muscle from an adult rabbit, 30 days after the cutting of the ipsilateral sciatic nerve. F, the same, but after treatment with 10^{-5} M physostigmine.

to conclude only grossly as to the size of enzyme activity. But it can be definitely stated that the muscle fibers showed the marked histochemical reactions when the myosincholinesterase activity computed for 1 gm of muscle was high (Kövér, Kovács et al., '57).

Picture A in figure 3 shows the color reaction of the gastrocnemius muscle from a normal two-day rabbit. Prior to incubation the section had been extracted at 0°C for two hours with KCl of 0.02 ionic strength. Comparing the reaction of this preparation with that of the muscle prepared under the same conditions, but not extracted (fig. 1, A) we see that such a low ionic strength has no substantial influence on the activity of either the neurogenic, or the intracellular cholinesterase. Picture B shows the cholinesterase activity of the same muscle, after extraction at 0°C for two hours with KCl having an ionic

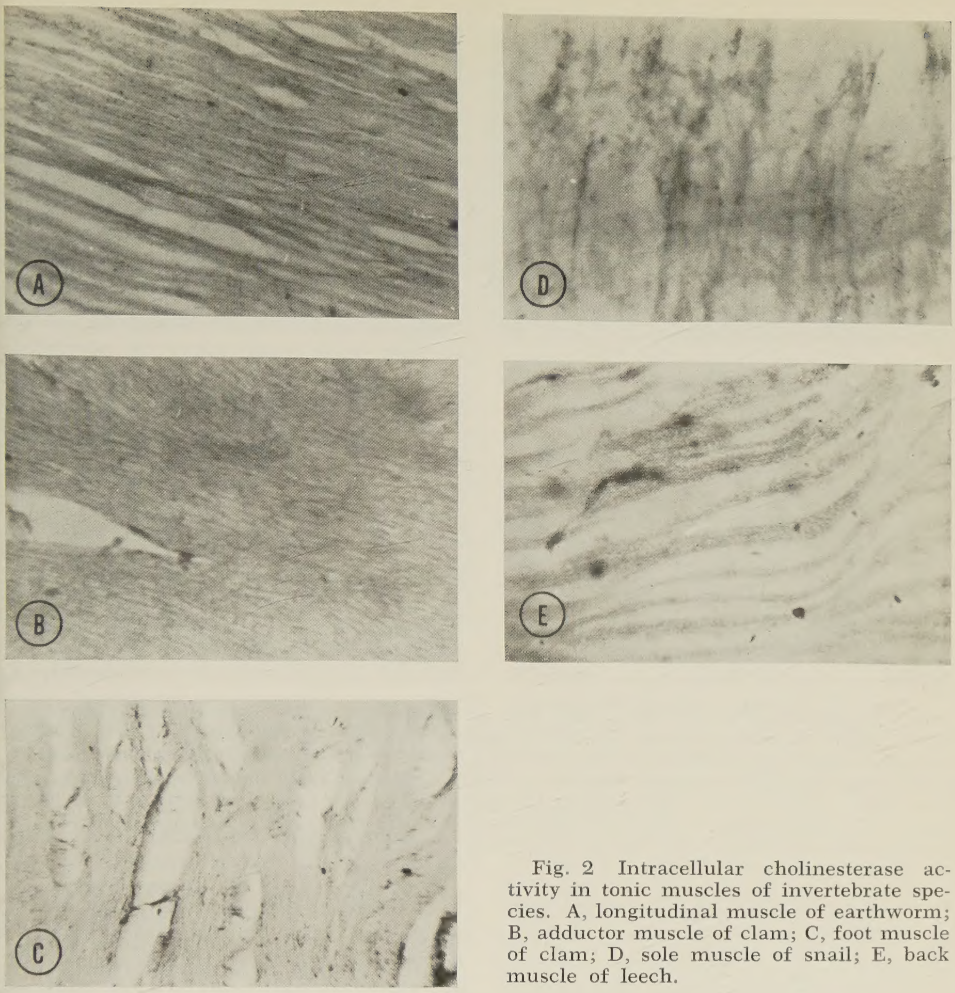

Fig. 2 Intracellular cholinesterase activity in tonic muscles of invertebrate species. A, longitudinal muscle of earthworm; B, adductor muscle of clam; C, foot muscle of clam; D, sole muscle of snail; E, back muscle of leech.

rength of 0.6. As compared with picture , the reaction of the end plates remained nchanged following extraction at high nic strength. A substantial change is sible in the muscle fibers. After the traction of myosin the muscle fibers ow hardly any visible reaction, in conast to the strong muscle fiber reaction the control preparation, the one treated ith low ionic strength. Similar results ave been obtained for denervated muscle, is illustrated by pictures C and D.

DISCUSSION

There is a considerable divergence tween the biochemical and histochemal evidence in the literature as to the cation of cholinesterase. The location and changes in the activity of acetylcholinesterase have been extensively studied by histochemical methods. Nevertheless, Portugalov ('55) and Gerebtzoff ('59) mentioned a "sarcoplasmatic" or intracellular cholinesterase activity of muscle. The question emerges: Is the intracellular cholinesterase activity of muscle really so low, as related to the acetylcholinesterase activity of the end plates, that it is not demonstrable by the methods used hitherto and the activity observed occasionally in muscles would really be a diffusion, preparative artifact of the histochemical reaction? This view is rather shaken by the evidence published by Couteaux and Nachmansohn ('40) that the pelvic part of the sartorius muscle of

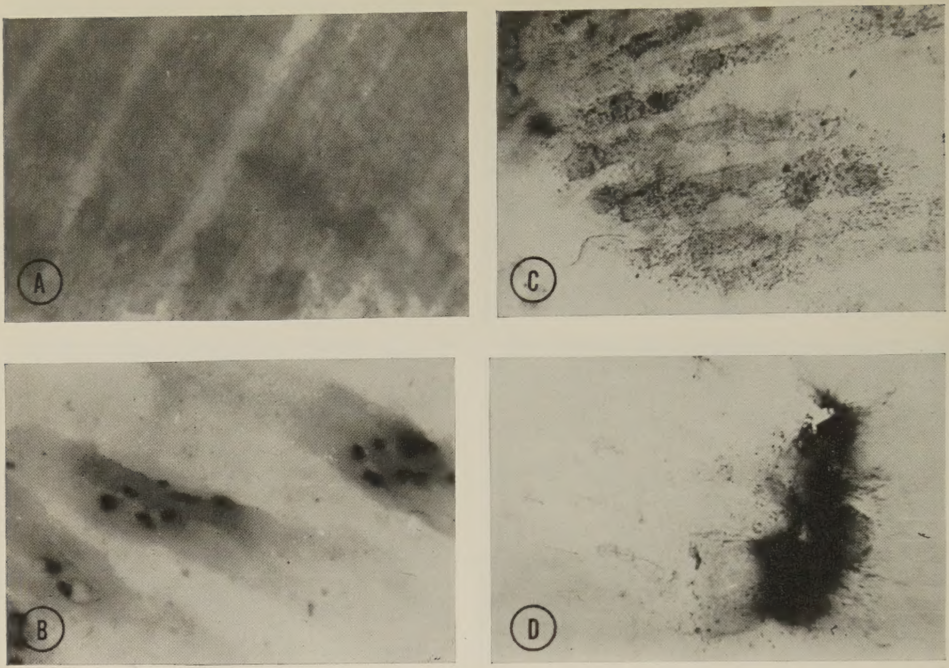

Fig. 3 The effect of treatment with KCl of different ionic strength on the intracellular cholinesterase activity. A, section prepared from the gastrocnemius muscle of a two-day-old rabbit, extracted at 0°C for two hours with KCl of 0.02 μ prior to the histochemical reaction. B, the same as A, but extracted with KCl of 0.6 μ. C, section from the gastrocnemius muscle of an adult rabbit, 30 days after cutting the ipsilateral sciatic nerve and treated as described under A. D, the same as C, but extracted with 0.6 μ KCl, under the same conditions. Magnification: objective Zeiss apo. 4 mm, ocular Leitz Periplan × 10.

the frog that contains no neural endings had a significant cholinesterase activity averaging, 0.4 mg acetylcholine/100 mg muscle/hour, which is about the quarter of the activity shown by the middle part containing also end plates. Our earlier experiments suggest that about half of the total cholinesterase activity is given not by cholinesterase concentrating at the neural end plates, but by the myosincholinesterase forming part of the contractile structure (Varga, Kövér, Kovács and Hetényi, '57a).

In the light of the evidence discussed above evaluation of the figures published by various authors leads to making interesting conclusions. Here we refer for example to a report by Kupfer and Koelle ('51) describing the changes observable by histochemical methods in the acetylcholinesterase activity of the end plates in the course of ontogenesis. If we compare their figure 25 with figure 30 it will be clear that in the former also the muscle fibers exhibit diffuse color reaction, wher
as in the latter they do not. Figure 25
a preparation made of the muscle of a
day-old rat; figure 30 is one made of th
muscle of an adult rat. This difference, a
related to our observations, tends to i
dicate that the diffuse color reaction in th
muscle from animals but a few days o
might be due to a substantially high
specific myosincholinesterase activity
such muscles. On the other hand, ther
is no visible color reaction indicative
cholinesterase activity in the muscle fro
the adult rat, because the activity of th
protein is reduced to about 1/10 in th
course of ontogenetical development. Als
we refer to figure 5 in a paper by Woh
fahrt ('55), that shows a diffuse color rea
tion in the muscle fibers of the denervate
rat muscle 30 days after operation, whi
no such reaction is visible in the musc
from the rat studied 12 days after dene
vation. In earlier experiments we showe

Varga, Kövér, Kovács and Hetényi, '57a) hat in rabbits myosincholinesterase acivity changes relatively in a small measire, or even tends to decrease in the eriod of from 8 to 18 days following deiervation, while the specific myosincholinsterase activity is significantly increased n the gastrocnemius muscles examined 25 o 29 or 46 days after denervation, to from hree to 4 times the normal activity. No loubt, we must be extremely careful when rying to compare the two observations, is different animals may give different esponses to the same intervention.

We mention also the similar results obained by Snell and McIntyre ('56), as vell as by Becket and Bourne ('57), which end to indicate that muscle fibers will how usually a color reaction, more or ess marked, when the activity of myosinholinesterase, thus that of the muscle holinesterase, is presumably high. We an confirm these reports by our own obervations.

As the research workers studied the locaion of cholinesterase usually not under he conditions optimal for the activity of nyosincholinesterase (owing to the causes iscussed in detail in the section on nethods), it could be expected that the reation of optimal conditions would eniance the demonstrability of the enzyme nd also the intensity of the color reaction. 'hus, in the first phase of our work we sed a modified Koelle-Friedenwald method or studying the location of cholinesterase. 'he figures show clearly that even in musles from the adult animal, color reaction s visible not only around the end plates, ut also in areas distant from them, i.e., vhere the presence of diffusion artifacts pointed out by Koelle and also by other uthors) cannot interfere anymore with he reliability of the results. Furthermore, can be stated that in every muscle in vhich we found high myosincholinesterase ctivity by biochemical methods (embrynic rabbit muscle, denervated gastrocneiius muscle of the rabbit, adductor muscle f clam, back muscle of leech, etc.) we ound also very marked histochemical rection in the muscle fibers. For example, n embryonic muscles the muscle fibers how an intense histochemical reaction, alhough in such muscle the structure of the nd plates is less differentiated.

We should like to mention here the chicken amnion experiments of Hoefke and Lüllman ('56), who found the muscle fibrils to show cholinesterase activity, although that tissue is not innervated. They also made the remarkable observation that the muscle fibrils of the chick amnion break down acetylcholine and butyrilcholine in an about equal measure. We stated earlier (Kövér and Kovács, '57) that myosincholinesterase can hydrolyze also butyrilcholine, though in an only lesser measure. Our data tend to indicate also that the specificity of myosincholinesterase changes corresponding to the structure and function of single muscles. For example, in the case of heart muscle myosincholinesterase hydrolyzed butyrilcholine in a greater measure than acetylcholine. In our opinion the above authors demonstrated not a new type of cholinesterase, but the cholinesterase activity of the myosin of amnionic muscle.

Recently Barrnett and Palade ('59) showed cholinesterase activity in M-band of muscle fibers using thiolacetic acid as substrate. Their results support our theory that intracellular cholinesterase activity of muscle is due to the activity of myosincholinesterase.

It is another question whether the intracellular cholinesterase activity demonstrated by histochemical methods by us can be ascribed to myosin activity, or, as stated by Portugalov and other authors, to "sarcoplasmatic" cholinesterase activity, or to the enzymatic property of some other protein.

Physostigmine inhibits equally the activity of acetyl- and myosincholinesterase *in vitro,* as it is visible in picture F of figure 1. At a concentration of 10^{-5} M physostigmine blocks the activity and the histochemical reactions of both the end plate and the intracellular cholinesterase. Thus, physostigmine alone is unsuitable for a qualitative separation of the two kinds of cholinesterase. In his experiments on frog tongue muscle, Portugalov ('55) employed pretreatment with prostigmine to differentiate "sarcoplasmatic" cholinesterase from end plate cholinesterase. It is known that under normal conditions the quaternary ammonium salts do not pass through the intact membrane structures. Thus the prostigmine employed at a suitable concentration in Ringer's solution will block only the cholinesterase on the surfaces and that at the postsynaptic junctions. In the preparations made after pretreatment with and washing out of prostigmine no histochemical reaction was visible in the sarcoplasm and at the end plates activity was greatly reduced. Of course, under such conditions even acetylthiocholine, which likewise possesses quaternary ammonium radical, does not enter the intracellular space. When, however, the sections were treated with acetone immediately after the treatment with prostigmine, the membrane loosened up, permeability increased and prostigmine inhibited or lessened cholinesterase activity at the sites mentioned only, while the intracellular "sarcoplasmatic" cholinesterase activity greatly increased, as a result of the increased permeability to acetylthiocholine. This fact practically rules out the possibility that the "sarcoplasmatic" cholinesterase activity would be a result of a diffusion of end plate cholinesterase.

To separate the two kinds of cholinesterase, and to study whether the "sarcoplasmatic," i.e., intracellular cholinesterase activity is truly the property of myosin, the contractile protein, we have extracted the sections with high ionic strength of KCl used for the extraction of myosin. The figures show clearly that only the diffuse reaction is abolished after the extraction of myosin while the cholinesterase activity of the end plates remains virtually unchanged. When evaluating the results, we should bear in mind that under the given conditions KCl of an ionic strength of 0.6 μ may extract also other proteins from muscle. But since at low ionic strength (0.02 μ) intracellular activity did not decrease, and as at present we do not know of any other cholinesterase that could be extracted from muscle fibers exclusively at high ionic strength, it seems likely that the intracellular cholinesterase activity we demonstrated is in fact myosincholinesterase activity.

SUMMARY

1. The authors have studied the intracellular cholinesterase activity of muscle fibers by histochemical methods. They have modified the procedure described by Koelle and Friedenwald. Raising the p of the incubation mixture, prolongation the duration of incubation, fixation wi formaldehyde (which increases the pe meability of the membrane to acetylthi choline) promote the demonstrability the intracellular cholinesterase distribut diffusely in the muscle fiber.

2. In striated rabbit muscle fibers t histochemically demonstrable cholineste ase activity is high in the early phase ontogenetical development and decreas with the advance of time.

3. In the denervated muscles of adu rabbit the intracellular cholinesterase a tivity increases 25 to 30 days after cuttin the sciatic nerve.

4. In tonic muscles of some low speci of the phylogenetical series (snail, eart worm, clam, leech, etc.) marked activi could be demonstrated by histochemic technique.

5. When the sections had been extract with KCl of high ionic strength prior making the histochemical reaction an thus the contractile protein had been e tracted from muscle, the intracellular a tivity of the muscle fibers decreased, whi in the controls which had been treat with KCl of low ionic strength intracell lar cholinesterase activity remained u changed. Treatment with either kind KCl left the activity of the motor end plat unaffected.

6. It is likely that the intracellular ch linesterase activity of the muscles is ide tical with the myosincholinesterase acti ity, because it was high just in tho muscles which had shown high myosi cholinesterase activity in our earlier e periments. Moreover, at present it is on the myosincholinesterase that is known occur intracellularly and can be extracte exclusively with high ionic strength.

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Investigations on the Physiological Role of Myosincholinesterase in Phylogenesis

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It has long been known that tonic muscles are characterized by a high acetylcholine content and by a high cholinesterase activity, while the tetanic ones show a relatively low cholinesterase activity and a low acetylcholine content (Ginetsinski and Michelson, '37, '38; Ginetsinski and Shamarina, '42; Leibson, '39, '43; Lissák and Endrőczi, '50; Rabinovskaia, '36; Rückert, '30). Many authors have suggested that the high acetylcholine sensitivity of tonic muscles would be correlated with the high cholinesterase activity (Marnay and Nachmansohn, '37; Nachmansohn, '38; Martini and Torda, '37; Strelina, '56; Strelina et al., '57; Torda, '39).

In our experiments on denervated and embryonic rabbit muscles we showed that in tonic skeletal muscles the specific activity of myosincholinesterase was several times higher than the activity of the myosin isolated from tetanic muscles of the adult rabbit (Varga et al., '57a,b). We found a rather close relationship between the acetylcholine sensitivity of muscles and the specific activity of myosincholinesterase, inasmuch as the acetylcholine sensitivity of the muscle is the highest when the specific activity of myosincholinesterase is the highest. Under the above conditions the adenosine triphosphatase showed a change similar in magnitude to that in cholinesterase activity, but in the opposite direction.

To elucidate in what measure the observations made in denervated and embryonic muscles are of general validity, we have made experiments with tonic muscles of some species in the phylogenetical series. We have also investigated the correlation between the increase of acetylcholine sensitivity, of the activity of myosincholinesterase, or the decrease of adenosine triphosphatase activity, on the one hand, and the ATP sensitivity of the muscles, on the other.

METHODS

For the enzyme activity assays myosin was prepared by two procedures. From muscles high in myosin [normal and denervated adult rabbit muscle, fish (*Amiurus nebulosus*), frog (*Rana esculenta*) and clam (*Anadonta cygnea*) muscle] myosin was prepared by the method of A. G. Szent-Györgyi ('51). From leech (*Hirudo medicinalis*), snail (*Helix pomatia*), earthworm (*Lumbricus terrestris*), as well as from embryonic rabbit muscle myosin was extracted with a 0.45 M KCl phosphate buffer at 0°C, then it was precipitated (Guba and Straub, '43). Both kinds of myosin were purified by several precipitations. To the myosin preparations thus obtained sufficient crystalline KCl was added to obtain an end concentration of 0.6 M. The pH of the preparations was adjusted to neutral by adding crystalline potassium bicarbonate, then two or three drops of toluol were added as a preservative. With both methods care had been taken to keep the temperature between 0 to 5°C throughout. The preparations were stored in a refrigerator until used. It is noted that the myosin from the leech back muscle could not be freed from contaminating dye, in spite of several precipitations.

The nitrogen content of myosin solutions was determined by the micro-Kjeldahl method; when computing protein content, the value of N was multiplied by 6.

Cholinesterase activity was measured once, at an acetylcholine concentration of 10^{-3} M, in the presence of a 0.01 M pH 8 veronal HCl buffer, at 37°C. Acetylcholine content was determined in aliquots taken from the incubation mixtures at 0 and 60 minutes, in a Havemann photometer (Hestrin, '49). Enzyme activity is expressed in micrograms of acetylcholine broken down by 1 mg of protein in one hour.

Adenosine triphosphatase activity was assayed at pH 9.1, in an incubation mixture composed of 0.03 M glycylsodium hydroxide buffer, 0.01 M KCl and 0.01 M tetrasodium adenosine triphosphate (end concentrations). The quantity of inorganic P released in 5 minutes was measured photometrically (Taussky and Shorr, '53). Adenosine triphosphatase activity is given in Q_p.

The acetylcholine sensitivity of the muscles was examined in an adequate salt solution and at a proper temperature; O_2 supply was provided for. The measure of acetylcholine sensitivity is given in terms of μg ACh/10 ml solution.

ATP sensitivity was examined in fibers extracted with aqueous glycerol. The muscles had been extracted for two days with equal volumes of water and glycerol in a refrigerator, then the glycerol was removed by washing with 0.15 M KCl solution. ATP sensitivity was examined in the presence of 0.15 M KCl, 4 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 6 mM ATP, at pH 7.2, at room temperature or at 38°C (Bendall, '53). ATP sensitivity is expressed by the size of the contraction shown by the same length of muscle under the same magnification, under the same load.

RESULTS

Muscles from such species of the phylogenetical series were used (clam foot and adductor muscle, leech back muscle, rectus muscle of the frog, sole muscle of the snail) as show high acetylcholine sensitivity and tonic function, respectively, according to the data in the literature. In our observation that tonic function or the high acetylcholine sensitivity are proportionate to the specific activity of myosin-cholinesterase, a similar correlation will have to be observable also in the above species of the phylogenetical series.

Figure 1 shows activity of myosincholinesterases isolated from muscles of different species. The single data represent the means of two to 5 determinations. It is visible that the activity of the myosincholinesterase from lower species is substantially higher than that of the myosin from tetanic rabbit muscle. For example, the fish and earthworm muscles showed nearly 9 times, the back muscle of the leech 6 times, the sole muscle of the snail 5 times, the adductor of the clam 4 times and the foot muscle of the same species two times higher activity. On the other hand, it is remarkable that the tonic and highly acetylcholine sensitive frog rectus muscle has a similarly low cholinesterase activity as the myosin from adult tetanic rabbit muscle. No difference was noted in cholinesterase activity between the myosin preparations isolated from the tonic frog muscle and from tetanic frog muscles. However, it should be emphasized also

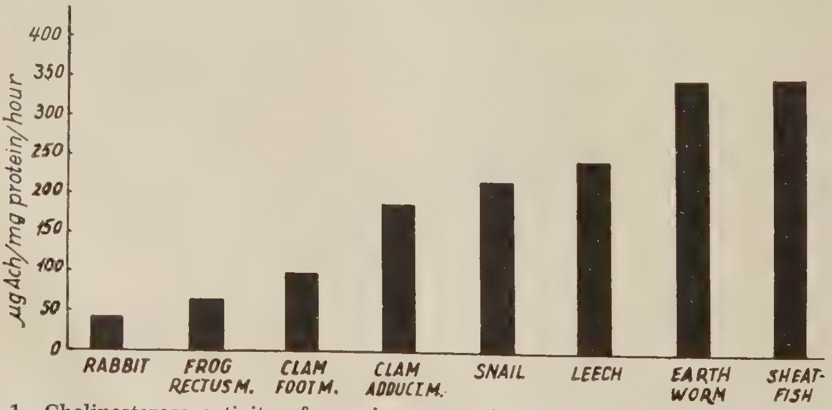

Fig. 1 Cholinesterase activity of myosin preparations isolated from muscles of various species. The height of the columns indicated the quantity of acetylcholine broken down in one hour by 1 mg of protein.

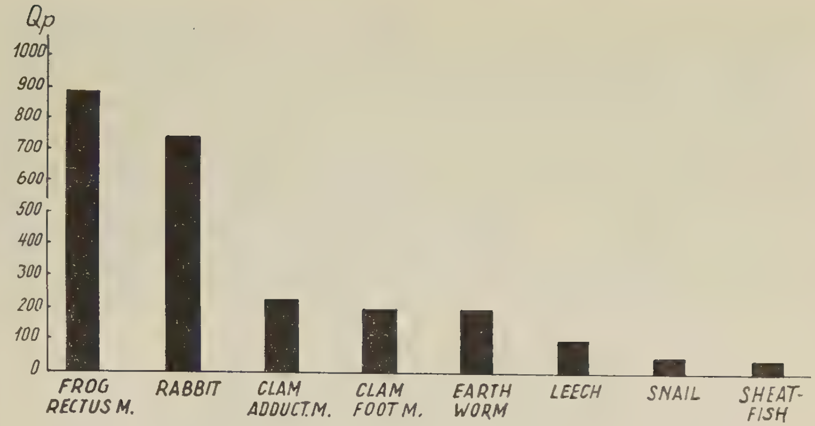

Fig. 2 ATP-ase activity of myosin preparations isolated from muscles of various species. The height of the columns indicates ATP-ase activity, in term of Q_p.

here that in the frog even the skeletal muscles possess a relatively high acetylcholine sensitivity.

The adenosine triphosphatase activities of these same myosin preparations are compared in figure 2. It is clear that every muscle showing high cholinesterase activity has low adenosine triphosphatase activity. Comparing the results presented in figure 1 with those in figure 2, it can be stated that as related to the enzyme activity values of the adult rabbit muscle myosin the decrease of the adenosine triphosphatase activity is comparable in measure to the increase of cholinesterase activity. On the other hand, the frog rectus muscle, which has low cholinesterase activity, shows a high adenosine triphosphatase activity, like the tetanic rabbit muscles.

In a few cases we have determined the actin-binding capacity of some myosin preparations. It has been found that the ability to bind actin changes parallel with he change in adenosine triphosphatase activity, i.e., the myosin preparations showing high cholinesterase and low adenosine triphosphatase activities can combine with much less actin than can the etanic muscles.

As it has been reported (Kuschinsky et al., '54; Weber and Portzehl, '52) that the contraction or the superprecipitation of he actomyosin sol induced by ATP are nseparably associated with splitting of ATP, it had to be investigated whether the esponse given to ATP by tonic muscles with low adenosine triphosphatase activity would become modified, corresponding to their enzyme activity.

In figure 3 are shown the acetylcholine and ATP sensitivities for normal and denervated rabbit psoas, as well as for embryonic psoas muscles. It can be seen that the acetylcholine sensitivity of the denervated psoas muscle increases more than 1000-fold, while the same muscle does not respond to ATP (after treatment with aqueous glycerol). In regard to ATP sensitivity, the embryonic psoas muscle behaves like the denervated adult one. Owing to technical difficulties we could not determine acetylcholine sensitivity in isolated embryonic psoas muscle; instead, we determined it in the gastrocnemius muscle, perfusing the hind limb as a whole and injecting acetylcholine.

In table 1 we compare the acetylcholine and ATP sensitivity of the muscles from the different species, as well as the specific activities of the myosincholinesterases (prepared from single muscles). The acetylcholine sensitivity values shown are those for untreated muscles, because extraction with water and glycerol resulted in a loss of acetylcholine sensitivity. It can be seen that the muscles with high myosincholinesterase and low ATP-ase activity (denervated or embryonic psoas and gastrocnemius muscles, leech back muscle, longitudinal muscle of the earthworm) show a marked sensitivity to acetylcholine, on the one hand, and do not respond to

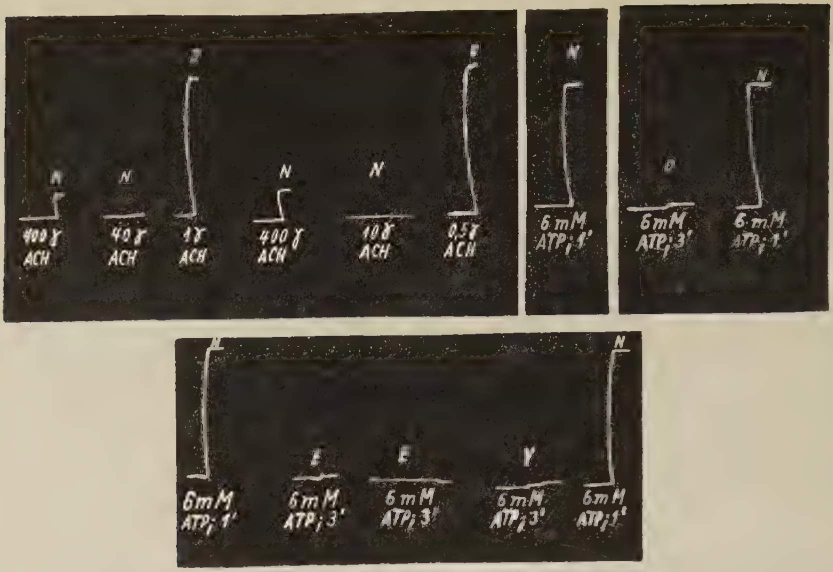

Fig. 3 Comparison of acetylcholine and ATP sensitivity in the case of normal and denervated adult rabbit's muscle and embryonic muscles. Symbols: N, normal; D, denervated; E, embryonic (prenatal); Y, two-day-old rabbit.

TABLE 1

Comparison of the ACh and ATP sensitivity of muscles and of the cholinesterase activities of myosins prepared from various species

Species	Muscle	ACh sensitivity	ATP sensitivity (6 mM ATP)	Activity of myosincholinesterase (μg ACh hydrolized by mg protein/ hour)
Rabbit (adult)	M. psoas	± 1–4 mg	+++	45.6
Rabbit (adult)	Denerv. M. psoas M. gastrocn.	+++ 0.1–1.0γ	±	529.0
Rabbit (2 days old)	M. psoas M. gastrocn.	+++ 0.1–1.0γ	—	388.0
Leech (*Hirudo med.*)	Dorsal muscle	+++ 0.01–0.1γ	—	239.7
Earthworm (*Lumbricus terr.*)	Longitud. muscle	++ 10–20γ	—	349.0
Frog (*Rana esc.*)	M. rectus	+++ 0.05–0.1γ	+++	41.3

ATP, on the other hand, in contrast with the normal tetanic muscles, which contract in the known way in response to ATP, but are less sensitive to acetylcholine. It has been mentioned beforehand that in regard to cholinesterase or ATP-ase activity the myosin from the frog rectus muscle (which is highly sensitive to acetylcholine and functions tonicly) behaves similarly to the tetanic muscle of the adult rabbit. The data in table 1 make it clear that unlike the other tonic muscles examined, this muscle responds to ATP like tetanic muscles do.

DISCUSSION

The uncertainty as to the correlation between cholinesterase activity or content and acetylcholine sensitivity that can be found in the literature is believed to be due to the fact that, not knowing the cholinesterase activity of myosin, some workers have correlated the changes in acetylcholine sensitivity with the changes in the total cholinesterase content or activity of the muscle. However, the proportions or the activity of the components giving the total cholinesterase activity of muscles may change under different experimental conditions. For example, it has been reported (Herrmann and Nicholas, '48; Robinson, '52a,b) that the contractile protein (i.e., myosin) content of embryonic muscles is low, then rapidly increases in the course of postnatal life. On the other hand, other experiments suggest that in the postnatal life neurogenic cholinesterase tends to become more and more concentrated to the areas of the neuromuscular junctions (Leibson, '39, '43).

According to Leibson, cholinesterase content and activity definitely increase during the first days of postnatal life. Our data (Varga et al., '57b) support this view. At the same time, acetylcholine sensitivity decreases with the advance of time at the onset of postnatal life (Kostojanc and Rabinovskaia, '35). These observations make it unlikely that a parallelism would exist between acetylcholine sensitivity and the changes in total cholinesterase activity. In a previous paper we have reported that about 50% of the total cholinesterase activity of the muscle is due to the activity of myosincholinesterase (Varga et al., '57a). We have also shown that the above increase of total cholinesterase does not reflect the changes taking place in myosincholinesterase activity. We have demonstrated that during the first days of postnatal life the specific activity of myosincholinesterase is greatly diminished with the advance of time, although both the total cholinesterase activity and the myosincholinesterase activity computed for 1 gm of muscle continue to increase. As in the early phase of postnatal life the amount of myosin increases in a greater measure than its specific cholinesterase activity decreases, this results in an increase of activity computed for 1 gm of muscle.

Following denervation of the muscle, on the other hand, the amount of myosin may decrease in a greater measure than the specific activity increases. For this reason, myosin cholinesterase activity/gram of muscle may decrease, in spite of an increase of specific activity. The above considerations make it clear how important it is to study the activity of the components of the structure one by one and to take into account the specific activity.

In earlier experiments we found a certain parallelism between the acetylcholine sensitivity of muscles and the specific activity of myosincholinesterase, inasmuch as the acetylcholine sensitivity of the muscles is usually the highest when the specific activity of myosincholinesterase is the highest. Our experiments with tonic muscles of some species of the phylogenetical series support the above view.

Studying the correlations between the cholinesterase and ATP-ase activity of muscles, a noteworthy correlation has been observed between the adenosine triphosphatase and cholinesterase activities of myosin prepared from tonic embryonic rabbit muscle. As compared to the myosin from tetanic muscle of the adult rabbit, in embryonic muscle the cholinesterase activity of myosin was relatively high, while the ATP-ase activity was low. During postnatal life the changes in the two activities are virtually the mirror images of one another. Similar observations have been reported (Székessyné and Herrmann, '58) for dystrophic muscles of rabbits suffering from E-avitaminosis. Our experiments on some species of the phylogenetical series seem to confirm also that view according to which the high cholinesterase activity is associated with a decreased ATP-ase activity.

As our experiments indicate the above mentioned rules to be of general validity, it had to be asked whether these divergences in enzyme activity might not involve profound changes, structural and functional. It is generally accepted that the energy for contraction is supplied in some way by the breakdown of adenosine triphosphate by the ATP-ase system. In muscles extracted with aqueous glycerol, in myofibrils, as well as in actomyosin sol *in vitro* experiments contraction could not be separated from the breakdown of ATP (Ashley et al., '56; Bendall, '53; Kuschinsky et al., '54).

Strelina, Ivanov and Zhukov ('57) have reported for striated frog muscles and for mollusc muscles that the tonic muscles low in actomyosin and highly sensitive to acetylcholine gave much smaller responses to ATP than did the tetanic muscles which contain much of actomyosin. In view of our observation that the myosin of tonic muscles has low ATP-ase and low actin-binding activity, it is apparent that the above authors' evidence suggest qualitative changes of myosin, in the first place. Our experimental results showed unequivocally that the change in the enzyme activity of myosin, notably the decrease of the ATP-ase activity of myosin, carries with it a modification of the response to ATP, insofar as the muscles showing low ATP-ase activity did not contract in our experiments, in response to ATP. Considering that the acetylcholine sensitivity of tonic skeletal muscles is the highest just when their ATP-ase activity or their response to ATP are the lowest, it may be assumed that in the case of all of these muscles there exists a contraction mechanism different from that in tetanic muscles and that under such conditions ATP may play no direct, decisive role in the contraction of these muscles. We think that in the native state the contractile structures of the muscles possessing such a high myosincholinesterase activity suffer changes in shape in response to acetylcholine, being acetylcholine receptor structures.

It is the behavior of the rectus muscle of the frog that does not fit into that hypothesis. But it is just the frog rectus muscle (a tonic muscle with high acetylcholine sensitivity) that we found to show high ATP-ase and low cholinesterase activity, similarly to tetanic adult rabbit muscles. The frog rectus muscle responds equally to ATP and acetylcholine. Experiments by other authors indicate that the tonic fibers of these muscles are innervated by the "small nerve system" (Kuffler and Vaughan Williams, '53). On grounds of all these we think it conceivable that in this case the tonic reaction of the muscle is realized not through a difference in structure, but through a difference in innervation. According to data in the literature the ability to bind actin and the ATP-ase activity are the properties of H-meromyosin, while cholinesterase activity is a property of L-meromyosin (Mihályi and Szent-Györgyi, '53; Varga et al., '54, '55). On this basis the low ATP-ase and high cholinesterase activity of myosin from tonic muscles, as opposed to the high ATP-ase and low cholinesterase activity of tetanic muscles, suggests the possibility that changes or differences in enzyme activity may be ultimately due to a change in the proportion of H- to L-meromyosin, within the myosin molecule. It is also possible that in lower muscle activity the L-meromyosin part plays a decisive role in contraction.

SUMMARY

1. Tonic muscles with high acetylcholine sensitivity usually have high myosin-cholinesterase activity.

2. The muscles with high myosincholinesterase activity show relatively low ATP-ase activity.

3. The muscles having high cholinesterase and low ATP-ase activity do not contract in response to ATP.

It is suggested that the recognition of the properties found to characterize tonic muscle—high myosincholinesterase and low ATP-ase activity, small actin-binding ability and low ATP sensitivity—may help to understand the characteristic physiological behavior (increased excitability, tonic contraction) of these muscles.

ACKNOWLEDGMENT

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The Free Running Period of the Bat Clock; Seasonal Variations at Low Body Temperature[1,2,3]

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In the study of biological rhythms it is ssential to differentiate between the unerlying biological rhythm(s) which may e driving many distinct processes in a ingle organism and whichever of these rocesses one happens to be measuring. In rder to get as much information about he *underlying* rhythm as possible it is nportant to be aware of the relationships etween the various features of the overt hythm which one measures and the coresponding features of the underlying hythm. As an example, there is clearly o necessary relationship between the amlitude of the overt rhythm of running ctivity and the amplitude of whatever ystem is timing its onset. Likewise, stoping an overt rhythm does not in any way ssure us that we have stopped the underying one.

Several features of the behavior of the vert rhythm do, however, give us infornation about the underlying rhythm (see ittendrigh et al., '58 for a discussion of ne significance of transients following esetting signals). One of the most direct f these is the free running period, i.e., ne period of the overt rhythm measured n the absence of external entraining ignals. If we assume that the overt hythm which we are assaying is in a teady state relationship with an underying rhythm which is driving it, then the eriod of the overt rhythm must be deendent on that of the underlying rhythm. Jnder these conditions a change in the eriod of the overt rhythm must reflect a hange in the period of the underlying ne.

Several experimental treatments, which night be expected to change the free runing periods of overt rhythms have failed o do so—indicating a somewhat surprisng stability of the underlying rhythm in this respect. For example, several *drugs* have been tested for possible effects on period length, and there are no convincing reports of any positive effect. Antimetabolites such as cyanide failed to produce a detectable change in the period of the rhythm of sporulation in *Oedogonium* (Bühnemann, '55) and neither thiouracil nor dinitrophenol altered the period of the activity rhythm of *Peromyscus* (Rawson, '56). In fact, the only clear case of "chemical" manipulation of the free running period is that reported by Bruce and Pittendrigh ('60) who studied the effects of heavy water on the *Euglena* rhythm. They find that the free running period of deuterated cells may be lengthened by as much as 5 hours. It is well known, from studies of a wide variety of organisms, that *temperature* has only a slight effect on the period of overt rhythms. The effect of temperature on the period length of most biological rhythms can be described by a Q_{10} of about 1.1 (Pittendrigh, '54; Bruce and Pittendrigh, '56; Rawson, '56; Hastings and Sweeney, '57). The length of the free running period is also slightly dependent on *light intensity* as Johnson ('39) first showed clearly in *Peromyscus*, and Aschoff's ('61) recent discussion extends this finding to a wide array of other organisms. However, the meaning of this

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action of light in terms of the mechanism of the rhythm remains obscure. An experimentally induced change in the free running period of an overt biological rhythm would be of great interest if it could be produced by an agent already known to have clearly defined physiological effects. This would allow us to make a concrete hypothesis about the physiology of the system being affected, the underlying rhythm itself.

The present study reports a large difference between the free running periods of "summer" and "winter" bats assayed under the same conditions. This difference is correlated with the annual cycle of hibernation in winter and activity in summer.

MATERIALS AND METHODS

The bats used in the present study were *Myotis lucifugus*, the most common bat in the Northeastern United States. Winter animals were collected from an abandoned mine in Hibernia, New Jersey, where there is a large hibernating colony. They were either used immediately or kept in hibernation in the laboratory until they were needed. Summer animals were collected during July and August from summer colonies in attics and barns and from behind shutters in the vicinity of Princeton, New Jersey.

A restraining device was built in which the bat's feet and tail were held between the felt-lined jaws of a brass clamp. The animal hung head down from this clamp and a glass ring was slipped over the bat's head and wings to keep the wings folded against the body. This ring was attached to an upright piece of lucite which held the clamp by means of a section of rubber tubing fitted over it which was then forced into a lucite ring permanently cemented to the upright lucite (see fig. 1). The hot junction of the thermocouple was inserted in the bat's rectum to a depth of 1.5 cm and the wires were taped to the clamp to hold the thermocouple in place. At the conclusion of each experiment, the thermocouple was examined and in no case was it found to have slipped out of the rectum. When the bat and thermocouple were in place, the whole apparatus was placed in a clear lucite cylinder, 5 inches in diameter by 6 inches high, with a tightly fitting cover. About an inch of water covered th floor of the lucite cylinder. This kept th humidity in the chamber high but th water did not touch the bat and it wa

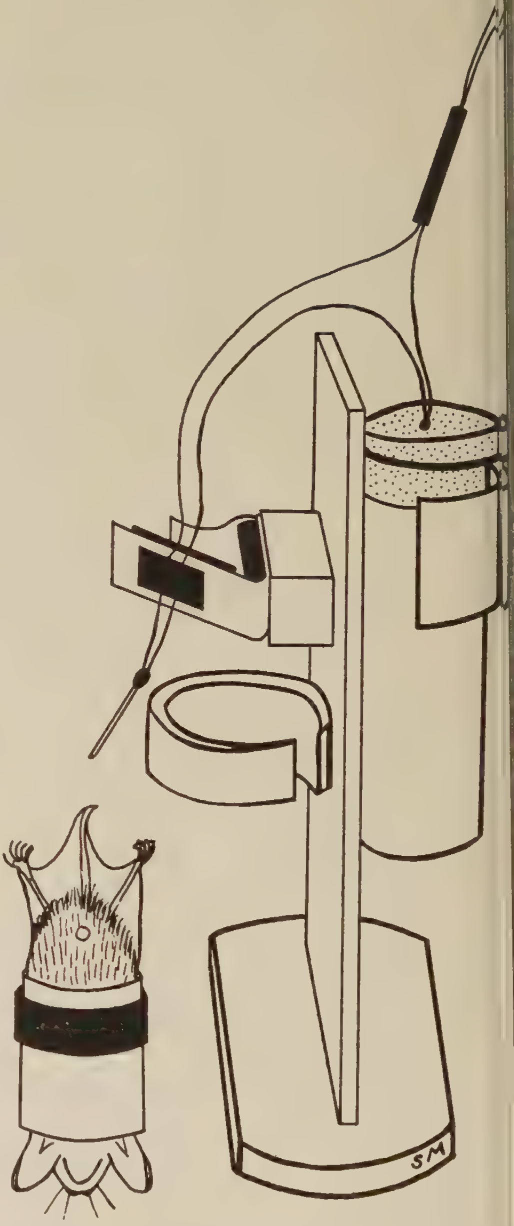

Fig. 1 Device for restraining a hibernating bat while a continuous record is made of the temperature difference between the bat (rectal probe) and his immediate environment (cotton filled vial containing reference junction).

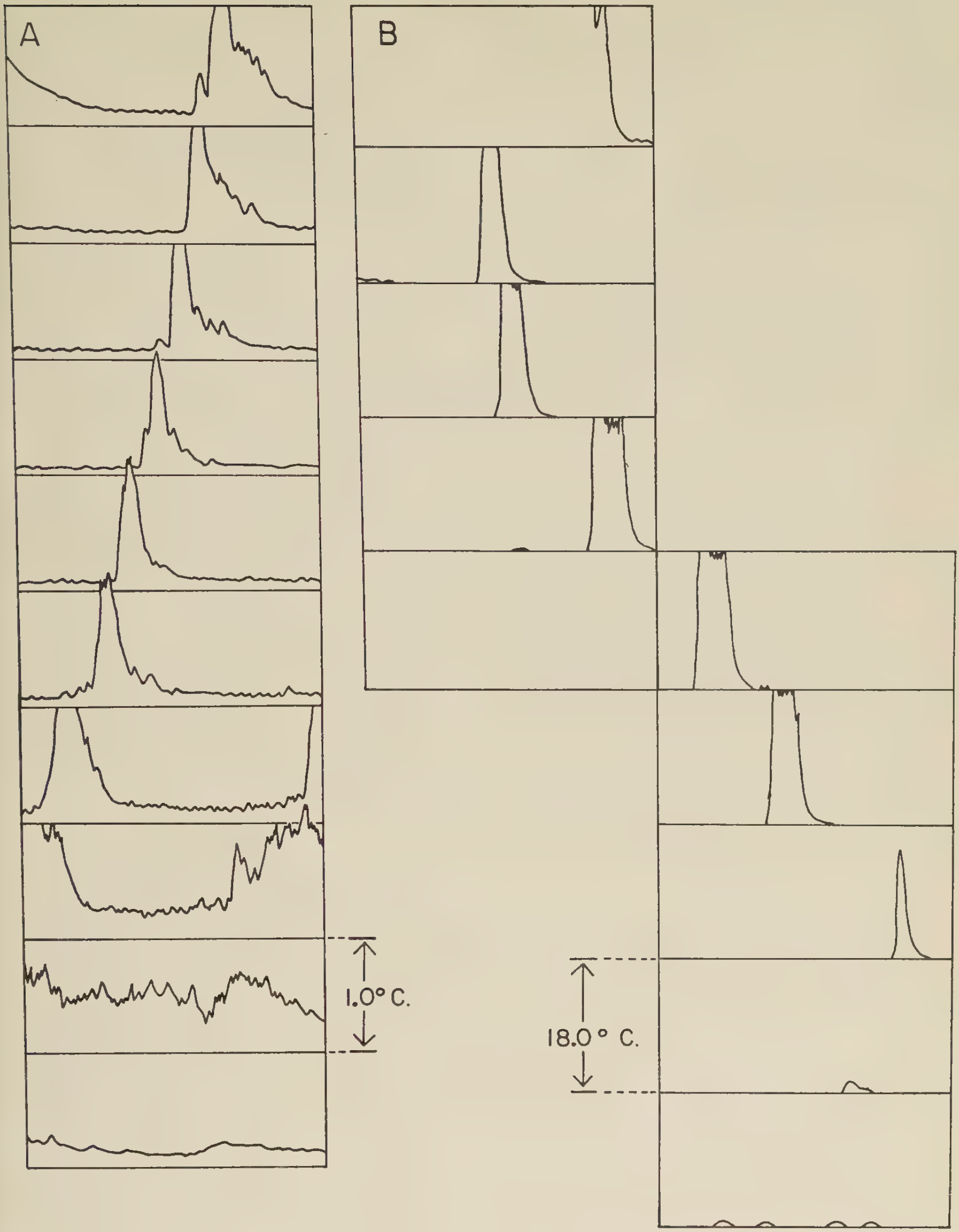

Fig. 2 Body temperature rhythms of (A) a short period summer bat (period about 22 hours) and (B) a long period winter bat (period about 30 hours from day 3 on). Both recordings were made in constant darkness and at a constant ambient temperature of 8.5°C. Each block is 24 hours with day 1 at the top. In *B* day 6 and subsequent days have been placed to the right of day 5 (on which no peak occurred) in order to make the period length more readily apparent.

not able to drink. A small hole was drilled in the cover of the cylinder to permit gas exchange. The cylinder containing water, bat clamp and bat was then placed in a precision temperature-controlled cabinet, in constant darkness and at the desired temperature.

The thermocouples were made from 30 gauge copper and constantan wire twisted together for about 2 mm and soldered at the tip. The hot junction, which was inserted in the bat's rectum, was cemented inside a fine glass capillary tube about 2.0 cm long and sealed at one end. The cold or reference junction was placed inside a cotton-filled vial to damp the small short-period fluctuations in air temperature and the vial was attached to the same upright piece of lucite which held the bat clamp.

A specially modified 4-channel Minneapolis-Honeywell recording potentiometer was used to record, in microvolts, the difference between the bat's rectal temperature and the temperature of the air immediately surrounding the animal in the lucite chamber. Using this potentiometer at its most sensitive setting, a difference of 1.25°C between the bat and its environment could be spread out over the entire 11-inch scale. Four bats at a time could be recorded continuously, each animal's temperature being recorded once every two minutes.

RESULTS

As reported previously, (Menaker, '59b) the Vespertilionid bats, *Myotis lucifugus* and *Eptesicus fuscus,* have an endogenously controlled rhythm of body temperature change when they are placed in a cold environment (3–10°C) at any season of the year. However, the period of this overt rhythm and therefore the period of the underlying rhythm which drives it is significantly different in summer and winter animals studied at the same low body temperatures. Summer bats, animals taken from the field during their summer season of activity, have rhythms of body temperature change with periods considerably shorter than 24 hours whereas winter bats, after a month or more of hibernation in either the field or the laboratory, have body temperature rhythms with periods longer than 24 hours.

Figure 2 shows continuous recordings of the body temperatures of a summer bat (A) and a winter one (B). Note the difference in the amplitude of the daily rise in body temperature as well as the difference in the period of the rhythm. The amplitude difference is a reflection of a difference in the physiology of body temperature regulation of summer and winter bats at low ambient temperatures (see discussion) and will be fully described in a later paper.

Figure 3 shows the distribution of period lengths of 7 summer and 16 winter bats, and the mean for each of these groups. The difference between the means is significant at the 0.002 level.[4] As the body temperatures at which these bats were assayed varied between 3 and 10°C, all the period lengths were corrected to 5°C, using a Q_{10} of 1.07, the highest Q_{10} found by Rawson in his study of the temperature compensation of bat activity rhythms. This correction affected the means only slightly and did not change the level of significance. It should be emphasized that the difference in period length between summer and winter bats cannot be explained on the basis of a direct temperature effect.

DISCUSSION

In constant darkness and at the same constant temperatures, bat rhythms show the widest range of free running periods of any organism so far studied; from about 21 hours to more than 28 hours. This range is beyond even the limits of entrainability of the circadian rhythms of many other vertebrates (Pittendrigh, '61). Further, as indicated above, the free running period of the bat rhythm, measured at low body temperatures, is correlated with some feature of the annual cycle of activity in summer and hibernation in winter.

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[4] The equality of the variances of the two samples was tested for statistically by the variance ratio or F-test. As the hypothesis of homogeneity of variance proved tenable, the "Student's" t-test was applied, and the difference between the means was found to be significant at the above level (Bailey, '59).

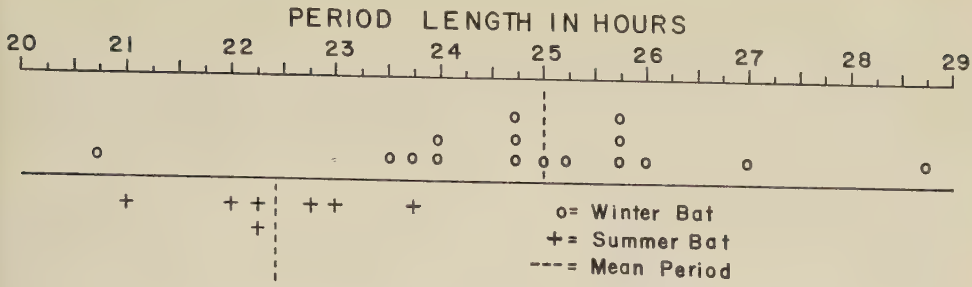

Fig. 3 A comparison of the distribution of free running periods of 7 summer and 16 winter bats. Each symbol ("○" for winter bats, "+" for summer bats) represents the period of the body temperature rhythm of an individual bat measured in constant darkness and at constant low (3–10°C) ambient temperatures. The periods are determined by eye fitting of slopes to raw data, such as is shown in figure 2. The number of days of data available for this determination from each animal varies between 4 and 21. The upper dotted line indicates the mean period of the winter group; the lower, that of the summer group.

The author feels that the endogenous nature of circadian biological rhythms has already been amply demonstrated. Additional confirmation, however, comes from the present study for it is difficult to conceive of an exogenous entraining variable which changes its period with the seasons.

The present data confirm and extend Rawson's ('56) finding of temperature compensation in the bat clock. The mean period of the activity rhythms of the four *Myotis lucifugus* and three *Eptesicus fuscus* in Rawson's study ('56) assayed at body temperatures which presumably varied between 25 and 37°C was between 20 and 22 hours whereas the mean period of the 7 summer bats in the present study is 22 hours and 25 minutes even though they were assayed at body temperatures more than 20°C lower. Rawson's data are compared with the summer bat data in the present study as Rawson's bats had been out of hibernation for some time. However, if we take 21 hours as the mean period length of Rawson's bats and compare this with the mean period of *all* the bats in the present study (24 hours, 10 minutes), assuming a 20°C difference in body temperature, we obtain a Q_{10} of about 1.07 which agrees well with Rawson's data and also with the numerous reports in the literature of the Q_{10}'s of the circadian rhythms of poikilotherms.

In attempting to account for the differences in period length between the rhythms of summer and winter bats one may not exclude the possibility that bats have an annual cycle of period change which is in no way related to hibernation. However, two considerations mitigate against this interpretation. First, a systematic dependence of period length on season has never been observed in any of the numerous non-hibernating organisms whose rhythms have been assayed at different seasons. Second, other experiments in this laboratory indicate that while winter bats are capable of raising their body temperatures from the hibernating level to approximately 39°C, summer animals, once they have dropped their body temperatures in the cold, cannot raise them more than 2 or 3°C unless passively warmed by an increase in ambient temperature (Menaker, '60). This summer-winter difference in arousability, which correlates well with the summer-winter period difference described above, suggests strongly that there is some physiological change associated with hibernation or preparation for hibernation which also exerts a marked effect on the period of the underlying rhythm.

Whatever the cause of the large differences in period length between the body temperature rhythms of summer and winter bats, the unique modifiability of the period of this endogenous rhythm may make the bat an unusually favorable animal in which to approach the physiological nature of the underlying rhythmic process or processes.

SUMMARY

Circadian rhythms of body temperature change in the bat, *Myotis lucifugus*, were assayed at constant, low ambient temperatures (3–10°C) and in constant darkness. The free running periods of these rhythms are significantly different in summer and winter animals. Summer animals have short periods (mean period of 7 animals = 22 hours, 25 minutes), whereas winter animals have long periods (mean of 16 animals = 25 hours). It is suggested that this seasonal variation in period may be the result of physiological changes, associated with preparations for hibernation, which also affect the biological clock.

The fact that the free running period of the *Myotis* clock is dependent upon season is regarded as new evidence that the clock is an endogenous mechanism, subject to change by the physiological state of the organism.

Taking into account the extremes of the summer and winter groups, the range of free running periods of *Myotis lucifugus* is the widest of any single organism so far investigated. Rawson's finding of temperature compensation in the bat clock is confirmed using a different assay of the rhythm, and is extended to include lower body temperatures than those in Rawson's study.

ACKNOWLEDGMENTS

I should like to acknowledge the invaluable advice and criticism given me in every phase of this study by C. S. Pittendrigh in whose laboratory this work was done. V. G. Bruce has also been most generous with his advice and his time and has made many pertinent suggestions.

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Electron Transport in Eggs, Developing Embryos and Adult Tissues of *Spisula solidissima*[1]

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It has been shown, by both spectroscopic and enzymatic assays, that a number of tissues from certain marine molluscs contain cytochromes similar to the cytochrome components of the terminal electron transport pathway of mammals (Ball and Myerhof, '40; Ghiretti-Magaldi et al., '57; Kawai, '59; Tappel, '60). Quantitative assays of the rate of oxygen consumption of whole eggs and developing embryos of the surf clam, *Spisula solidissima*, (Sclufer, '55) have also indicated that these marine eggs have an appreciable respiratory rate which is approximately doubled in 6-hour-old developing embryos. The purpose of the present study was to examine, in more detail, the terminal electron transport pathway in eggs, developing embryos and adult tissues of *Spisula solidissima*, and to determine whether or not qualitative or quantitative changes occur in this system of enzymes during embryonic differentiation. These experiments indicate that eggs, embryos and adult tissues of *Spisula* contain cellular particles resembling mitochondria in their terminal electron transport pathway. In addition, a second particulate fraction, which resembles liver microsomes in its oxidative enzyme pattern and absorption spectrum, has been isolated from unfertilized *Spisula* eggs. No qualitative or marked quantitative changes in the pattern of terminal electron transport has been observed during early embryonic differentiation, but adult heart muscle appeared to differ significantly from eggs and developing embryos in its succinic dehydrogenase activity and cytochrome complement.

EXPERIMENTAL PROCEDURE

The experiments described below were carried out with *Spisula solidissima* collected during the summer months in the Woods Hole area. Eggs were harvested from *Spisula* ovaries and washed 6 or 7 times with approximately 200 ml of freshly filtered sea water. In each washing the eggs were allowed to settle through a 4 to 5 cm depth of sea water for 10 minutes and the sea water and immature eggs were then siphoned off. The washed egg volume was determined by centrifuging the eggs for 30 seconds at 1000 × g. For fertilization, the eggs were suspended in 100 volumes of sea water in wide finger bowls approximately 4 to 5 cm deep and 10 to 12 cm in diameter. From 0.4 to 0.8 ml of a 1 to 50 dilution of concentrated fresh sperm in sea water was added dropwise to each 200 ml of egg suspension with vigorous stirring to give a slight excess of active sperm over eggs. By this procedure 95 to 100% nuclear breakdown and from 75 to 95% synchronous division were usually achieved as described by Allen ('53).

For growth of embryos, the fertilized eggs were permitted to develop at 23° in filtered sea water, with changes of sea water at 15 minutes after fertilization and, for older embryos, again at 10 hours after fertilization. The normal pattern and chronology of *Spisula* development (Costello et al., '57) were observed in these cultures.

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[1] Preliminary reports of this work have appeared (Strittmatter and Strittmatter, '60; Strittmatter et al., '60). This investigation was supported by research grants RG-6277 and RG-7260 from the United States Public Health Service and grant G-6136 from the National Science Foundation. Each author was the recipient of a Lalor Fellowship, P.S. during the summer of 1959 and C.F.S. during the summer of 1960.

Separation and isolation of 4-cell Spisula embryo cells. *Spisula* embryos at the 4-cell stage were harvested by centrifuging at 1000 × g for 30 seconds, washed twice with cold 0.52 M NaCl, 0.02 M Tris acetate buffer, pH 8.0, (NaCl) to remove calcium, then suspended in cold 0.7 M sucrose, 0.02 M Tris acetate buffer, pH 8.0 (SM). On prolonged standing in this medium, the cells of the embryos dissociated slowly, but complete dissociation was rapidly achieved by adding 0.0005 M EDTA[2] and gently agitating the suspension. The separated cells retained a normal appearance and were capable of further cleavage. The suspension of dissociated cells, containing large D cells and a mixture of the small A, B and C cells, was diluted with NaCl medium to give a 60 SM:40 NaCl mixture. A 2-ml aliquot of this mixture was layered over 10 ml of 75 SM:25 NaCl in a 15-ml tube and centrifuged at about 70 × g for three minutes. A 2-ml fraction drawn off from the gradient boundary contained an 80 to 90% pure mixture of A, B and C cells in about 20% yield, while a 2-ml fraction from near the bottom of the tube contained 90% D cells in about 20% yield. The purity of each fraction could be increased by a second centrifugation.

Preparation of homogenate and cell fractions. A single general procedure, with some slight modifications, was used for preparing homogenates from eggs, embryos at various stages of development and adult tissues. Eggs and embryos were collected by centrifuging the suspensions for 30 seconds at 1000 × g to determine the volume of packed material, and then washed two times with 5 volumes of cold 0.52 M NaCl containing 0.02 M Tris acetate and 0.005 M EDTA, pH 8.0, using the same centrifugation procedure. In the case of adult tissues the material was excised from freshly opened clams, washed in 0.52 M NaCl containing 0.02 M Tris acetate and 0.005 M EDTA, pH 8.0, and then blotted, weighed and minced. All preparations were homogenized at 0 to 5° with 10 volumes of sucrose medium (0.70 M sucrose, 0.02 M Tris acetate and 0.005 M EDTA, pH 8.0) per ml eggs or per gm tissue in a Potter-Elvehjem glass homogenizer fitted with a teflon pestle. To obtain 85 to 100% cell breakage required from 6 to 10 strokes of the homogenizer for eggs, from 10 to 60 strokes for embryos at various stages of development and 15 to 30 strokes for adult tissues.

Centrifugal fractionation of whole homogenates was carried out using a modification of the procedure of Schneider ('48) for homogenates derived from mammalian tissues. A fraction, N, was obtained by centrifuging the homogenate at 600 × g for 10 minutes. The supernatant fluid was then centrifuged at 13,000 × g for 12 mintues to obtain a particulate fraction, M. Finally, the supernatant fluid from the second centrifugation was diluted with one volume of cold distilled water and centrifuged at 100,000 × g for 90 minutes, yielding a small particulate fraction, R, and the supernatant fluid, S. All precipitates were resuspended in three to 5 volumes of the sucrose medium used to prepare the homogenate.

Assays. Enzymatic assays and spectral measurements were made with a calibrated Beckman model DU spectrophotometer with a photomultiplier attachment. The temperature was maintained at 25° by circulating water from a constant temperature bath through Beckman thermospacers. The rate of oxidation of DPNH was followed at 340 mμ to assay DPNH-ferricyanide reductase; the rate of reduction of cytochrome *c* was followed at 550 mμ to assay DPNH-, TPNH- and succinate-cytochrome *c* reductases; and the oxidation of reduced cytochrome *c* was followed at 550 mμ to assay cytochrome oxidase. The change in absorbancy was recorded in each case at 15 second intervals for at least three minutes, and activity calculated for the period of linear change in absorbancy. The assays were carried out in aerobic microcells in 0.20 ml 0.1 M Tris acetate, 0.001 M EDTA buffer, pH 8.1, containing the enzyme, electron donor, electron acceptor and at times other additions as indicated. For the spectrophotometric studies, the pellets of the particulate fractions obtained by the fractionation procedure described above were dispersed in 5% desoxycholate, 0.1 M Tris acetate buffer, pH 8.1, allowed to stand for 10 minutes at room temperature and clarified by centrifuging for 60 minutes at 100,000 × g. Appropriate dilutions of these preparations in 5% desoxycholate were then used for the spectral measurements.

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[2] EDTA refers to ethylenediaminetetraacetic acid.

The Nadi and Janus green staining reactions carried out with various preparations were modifications of procedures described previously (Ries, '37; Hogeboom et al., '48). The DPNH and cytochrome *c* were obtained from the Sigma Chemical Company. Reduced cytochrome *c* was prepared by adding limiting amounts of sodium hydrosulfite to an oxidized cytochrome *c* solution until 90% reduction of the cytochrome was achieved.

RESULTS

Electron transport activities of unfertilized Spisula eggs. Table 1 shows that the unfertilized eggs of *Spisula solidissima* contain a spectrum of oxidative enzyme activities similar in pattern to the terminal electron transport system found in mammalian tissues. The complete inhibition of succinate cytochrome *c* reductase by the antimalarial, SN5949, or by antimycin A and of cytochrome *c* oxidase by KCN emphasize this similarity and suggest that no appreciable amount of terminal electron transport occurs by an oxidative pathway involving a direct flavoprotein interaction with oxygen.

Fractionation of homogenates of *Spisula* eggs by differential centrifugation, as described in experimental procedure, yielded three particulate fractions corresponding to the nuclear (N), mitochondrial (M) and microsomal (R) fractions that would be obtained from mammalian liver using the same procedure. Gross and microscopic examination of the three particulate fractions obtained from the egg homogenates indicated that each is a heterogeneous mixture of several particle types varying in size, shape and pigment content. However, the M fraction corresponding to the mammalian mitochondrial frac-

TABLE 1

Electron transport activities of unfertilized Spisula egg homogenates and particulate fractions

The assay systems were as described in experimental procedure, and contained either 10^{-4} M DPNH, 10^{-4} M TPNH, 10^{-3} M succinate or 4×10^{-5} M reduced cytochrome *c* as electron donor and 2×10^{-4} M potassium ferricyanide, 5×10^{-5} M oxidized cytochrome *c* or oxygen as electron acceptor.

Cell fraction	Electron donor	Electron acceptor	Other additions[1]	Activity[2] µmoles electrons per minute per ml eggs
Homogenate	DPNH	Potassium ferricyanide	—	25 –31
Homogenate	DPNH	Cytochrome *c*	KCN	10 –13
Homogenate	TPNH	Cytochrome *c*	KCN	1.1 – 1.3
Homogenate	Succinate	Cytochrome *c*	KCN	0.15– 0.20
Homogenate	Succinate	Cytochrome *c*	KCN, SN5949	0.00
Homogenate	Succinate	Cytochrome *c*	KCN, Antimycin A	0.00
Homogenate	Cytochrome *c*	Oxygen	—	3 – 5
Homogenate	Cytochrome *c*	Oxygen	KCN	0.00
Fraction N	DPNH	Potassium ferricyanide	—	4 – 5
Fraction M	DPNH	Potassium ferricyanide	—	11 –13
Fraction R	DPNH	Potassium ferricyanide	—	4 – 5
Fraction N	DPNH	Cytochrome *c*	KCN	2.8 – 3.2
Fraction M	DPNH	Cytochrome *c*	KCN	6.3 – 7.7
Fraction R	DPNH	Cytochrome *c*	KCN	1.5 – 2.0
Fraction M	Cytochrome *c*	Oxygen	—	2.4 – 2.8
Fraction R	Cytochrome *c*	Oxygen	—	0.00
Fraction M	Succinate	Cytochrome *c*	KCN	0.11– 0.13
Fraction M	Succinate	Cytochrome *c*	KCN, SN5949	0.00
Fraction R	Succinate	Cytochrome *c*	KCN	0.00

[1] The concentration of KCN was 10^{-3} M; SN5949, 8×10^{-6} M; antimycin A, 10^{-6} M.

[2] These values indicate the range of activities obtained in assays of two to 5 preparations, each derived from the pooled eggs from several dozen *Spisula*.

tion contained a high concentration of particles which gave positive Janus green staining and cyanide-sensitive Nadi reactions, characteristic for mitochondria, while the R fraction was negative for both staining reactions. Several oxidative activities of these three cellular fractions are shown in the lower part of table 1. The cytochrome oxidase and succinate cytochrome *c* reductase activities are concentrated in the M fraction and completely absent from fraction R. However, both fractions M and R do contain DPNH cytochrome *c* reductase and ferricyanide reductase activities.

Difference spectra of fractions M and R. The difference spectra of desoxycholate suspensions of fractions M and R in the presence and absence of sodium hydrosulfite are shown in figure 1. The M fraction, curve 1, shows a cytochrome difference spectrum similar to that of mammalian mitochondria. Absorption maxima corresponding to the α peaks of cytochromes $a + a_3$ and *b* are clearly discernible at about 600 mμ and 562 mμ respectively, while the presence of cytochromes $c + c_1$ is indicated by the shoulders on the low wavelength side of the α peak, of the β peak and of the Soret peak of cytochrome *b*. The difference spectrum of the R fraction is similar to the difference spectrum of mammalian microsomal cytochrome b_5 (Strittmatter and Ball, '54).

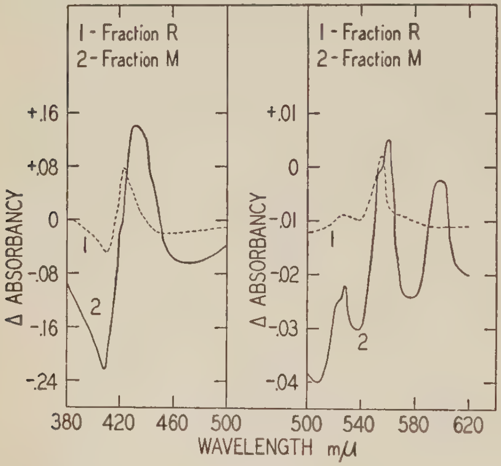

Fig. 1 Difference spectra of fractions R and M from unfertilized *Spisula* egg homogenates. Absorbancy readings were taken at 5-mμ intervals over the entire wave-length range and at 2-mμ intervals at each absorption maximum or minimum. The preparation of desoxycholate extracts is described in Experimental Procedure. Each difference spectrum represents the difference between the spectra of preparations reduced with sodium hydrosulfite and untreated. Curve 1 is the difference spectrum of fraction R derived from 5 ml eggs and suspended in 1.0 ml desoxycholate solution; curve 2, that of fraction M derived from 1 ml of eggs and suspended in 1.0 desoxycholate solution.

Electron transport activities in developing Spisula embryos and adult tissues. Cytochrome *c* oxidase, succinate cytochrome *c* reductase, and DPNH cytochrome *c* reductase activities were assayed in homogenates from unfertilized eggs, fertilized eggs, developing embryos and adult tissues. As table 2 shows, there were no marked changes in any of these activities at several stages in the embryonic development of *Spisula* up to 22-hour swimming forms. The small variations observed could have resulted from biological variation, from differences in homogenizing time required to achieve complete cell breakage or from differences in the ratio of "active protoplasm" to wet weight at various developmental stages. In the case of embryos at the second division, the technique described in Experimental Procedure was used to isolate the large D cells and a mixture of A, B and C cells. These separated cell types also did not show any marked differences, from each other or from the whole embryo preparation, in their content of the enzyme activities examined.

While the enzyme activities of the adult liver homogenates assayed here were very similar to those of *Spisula* eggs, the succinate cytochrome *c* reductase activity of heart muscle homogenates showed an 8- to 10-fold elevation, based either on the absolute activity of this enzyme in other tissues or eggs, or on the activity relative to the cytochrome *c* oxidase activity or DPNH cytochrome *c* reductase activity. Preparations from heart muscle of the particulate fraction M, in which these activities are concentrated, showed a similarly high ratio of succinate cytochrome *c* reductase to cytochrome oxidase activity, in contrast to the low ratio (about 1.25) found in both homogenate or fraction M of *Spisula* eggs (table 1).

TABLE 2

Electron transport activities of unfertilized Spisula eggs, embryos and adult tissues

Tissue	Activity[1] (μmoles electrons per minute per ml eggs and embryos or per gm tissue)			Number of preparations assayed
	Succinate cytochrome *c* reductase	Cytochrome *c* oxidase	DPNH cytochrome *c* reductase	
Unfertilized eggs	0.17	4.1	12.2	5
Eggs, 30 minutes after fertilization	—	4.7	13.0	2
Second division embryos	0.23	3.1	10.2	2
Second division A, B, C cells	0.17	3.8	10.5	2
Second division D cells	—	3.7	11.9	1
22-hour-old swimming forms	0.20	2.2	—	2
Adult liver	0.15	2.9	—	2
Adult heart	1.81	3.6	14.8	2

[1] Measured in assay systems described in experimental procedure with 10^{-4} M DPNH, 10^{-3} M succinate or 4×10^{-5} M reduced cytochrome *c* as electron donor and 5×10^{-5} M cytochrome *c* as electron acceptor. For the succinate and DPNH assays 10^{-3} M KCN was present. Each value tabulated represents the mean of multiple assays on the number of preparations indicated.

Comparison of the difference spectra of the M fractions of Spisula eggs and heart muscle. The difference spectra of the cellular particulate fractions M from eggs (fig. 1) and from heart muscle (fig. 2) show no qualitative differences. Both appear to contain cytochromes *b*, $a + a_3$ and $c + c_1$ The notable difference between these two spectra is the apparently higher concentration of cytochrome $c + c_1$ relative to the cytochrome *b* in the heart preparation. Whereas the cytochrome $c + c_1$ peaks are barely discernible in the egg preparation, the α peak of cytochrome $c + c_1$ in the heart particle preparation is quite distinct and higher than the cytochrome *b* peak.

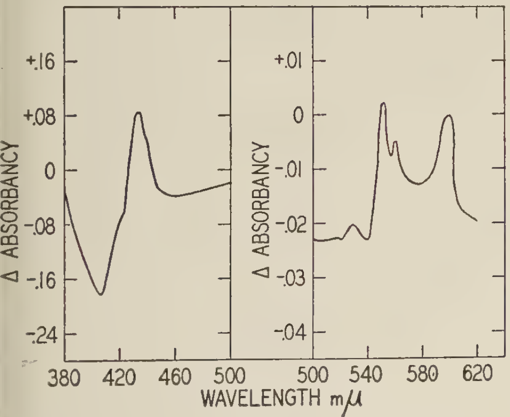

Fig. 2 Difference spectrum of fraction M from heart muscle homogenates. Absorbancy readings were taken at 5-mμ intervals over the entire wave-length range and at 2-mμ intervals at each absorption maximum or minimum. The preparation of the desoxycholate extract is described in Experimental Procedure. The difference spectrum represents the difference between the spectra of the preparation reduced with sodium hydrosulfite and untreated. The spectrum is that of fraction M derived from 1 gm of heart muscle and suspended in 1.0 ml of desoxycholate solution.

DISCUSSION

The characterization of the terminal electron transport system in *Spisula* eggs by both enzymatic assays and spectral analysis indicates that this oxidative pathway is very similar to those found in mammalian tissues (Strittmatter and Ball, '54), chick embryos (Brand et al., '60), and other marine eggs (Krahl et al., '41; Rothschild and Tyler, '58). The particular cellular particle containing this enzyme system also resembles mammalian mitochondria in sedimentation characteristics, staining properties and cytochrome content. Smaller particles sedimenting with the characteristics of the mammalian microsomal fraction were also found to contain, like microsomes, several reductase activities but negligible cytochrome oxidase activity and to possess a difference spectrum very similar to that of liver microsomes (Strittmatter and Ball, '54). The presence of a difference spectrum resembling that of liver microsomal cytochrome b_5 in unfertilized eggs is of particular interest because cytochrome b_5-like components have not been reported previously in eggs or in embryonic tissues except in the liver of 14-day-old chick embryos (Brand et al., '60) and in insect larvae (Pappenheimer and Williams, '54).

The three oxidative enzyme activities chosen to characterize the electron transport system during development—succinate cytochrome *c* reductase, DPNH cytochrome *c* reductase and cytochrome oxidase—failed to show any marked changes, either absolute or relative to each other, following fertilization of *Spisula* eggs or in embryonic development up to 22-hour swimming forms. Thus the extensive cell division and differentiation during this period apparently entailed neither a significant increase in the total content of functional respiratory particles ("mitochondria") in the embryo nor a differentiation of the respiratory particles with respect to these enzymatic components. The respiratory rate reported for *Spisula* eggs (Sclufer, '55) is equivalent to about 1 μmole electrons per minute per ml eggs, and rises gradually during early development to twice this level in 7-hour swimming blastulae. The data presented here therefore indicate that unfertilized eggs already possess, and embryos maintain, certain potential capacities for terminal electron transport which are greatly in excess of the levels necessary to account for the reported respiratory rates of eggs and early embryos.

The marked elevation of succinate cytochrome *c* reductase activity in heart muscle preparations, both on an absolute basis and relative to cytochrome oxidase activity, when compared to egg, embryo and liver preparations, suggests that a differentiation of mitochondrial oxidative enzyme complement may occur in later development. It may be significant that, except in heart, the succinate cytochrome *c* reductase activity was of a lower order of magnitude than the other respiratory activities examined, and was therefore more likely to have been a limiting factor in oxidative metabolism. The apparent difference in relative concentrations of cytochrome components between fractions M from heart and eggs may also reflect mitochondrial differentiation; however, this observation is accorded less assurance because of the uncertainties in quantitative estimation of small changes in cytochrome content from difference spectra of desoxycholate preparations from concentrated tissue fractions.

The present study suggests several points for further investigation. First, since a dramatic change in oxidative enzyme level or pattern from that in eggs was seen only in a specific adult organ, examination of embryonic tissues during organogenesis may localize the beginning of the apparent enzymic differentiation. Second, since it was the flavoprotein to cytochrome, antimycin A-sensitive part of the electron transport system that showed change, a focus on other activities at this oxidation level may reveal additional enzyme alterations during development. Finally, a definitive characterization of the type of enzyme differentiation studied here will involve consideration of such problems as the relation between observed assay activities and physiologic activities and the ability to distinguish activation of preformed enzyme from increased enzyme formation.

SUMMARY

1. The activities of a number of enzymes of the terminal electron transport system in homogenates of unfertilized eggs of *Spisula solidissima* have been determined.

2. Fractionation by differential centrifugation yielded two particulate fractions, one resembling mammalian mitochondria in enzymatic activities, staining characteristics and cytochrome content and another resembling mammalian liver microsomes in reductase activities and cytochrome spectrum.

3. No marked changes in three oxidative enzyme activities were observed in eggs 30 minutes after fertilization, in embryos at the 4-cell stage or in 22-hour-old swimming forms. With the exception of succinate cytochrome *c* reductase all of the activities were far in excess of the reported respiratory rate of *Spisula* eggs (Sclufer, '55).

4. A marked increase in succinate cytochrome *c* reductase activity both absolutely and relative to other oxidative enzyme activities was observed in preparations from adult heart compared to those from unfertilized eggs.

ACKNOWLEDGMENT

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Ca^{45} Uptake by Dog Erythrocytes Suspended in Sodium and Potassium Chloride Solutions[1]

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Interactions between inorganic cations re well known in biology but the basic mechanisms underlying such phenomena emain largely undetermined. One explanation for the interaction between calcium and sodium ions is that the similarity in ionic radii of these ions may result in a competition for transport channels in biological membranes (Mullins, '56). The greater accumulation of radiocalcium by isolated frog hearts perfused with K-Ringer's in contrast to Na-Ringer's (Niedergerke, '59) appears to support this hypothesis. The present report is concerned with a similar finding in dog erythrocytes suspended in either isotonic KCl or NaCl. In addition, the effects of storage and of pH on Ca^{45} uptake are described.

MATERIALS AND METHODS

Adult mongrel dogs anesthetized with Nembutal (sodium pentobarbital) were bled through the femoral artery and to each 100 cm^3 of shed blood was added 10 mg of Sodium Heparin (Lederle) to prevent coagulation. In each experiment, 15 cm^3 portions of blood were centrifuged at top speed in an International Clinical Centrifuge for 10 minutes. The plasma, buffy coat, and top layer of red cells were removed, leaving behind 4 cm^3 of packed erythrocytes. The cells were washed twice with 9 volumes of either isotonic NaCl or KCl and were then suspended in a final volume of 10 cm^3 of either salt solution.

One and one-half cubic centimeters of the blood suspension was placed in 20-cm^3 beakers to which had been added 1.0 cm^3 of radioisotope medium containing either isotonic NaCl or KCl, Tris buffer at pH 7.4 (12 mM/l), CaCl$_2$ (0.012 mM/l), and Ca^{45} (0.25 µC, Oak Ridge). Thus, one set of beakers differed from the other only in that it contained Na rather than K ions. Samples were prepared in duplicate. The beakers were shaken in a Dubnoff Metabolic Shaker at a rate of 96 oscillations/minute for 30 minutes at room temperature. pH was recorded with a Beckman combination electrode no. 39183 connected to a model G pH meter.

Following the incubation, the blood suspension was transferred to graduated tubes and centrifuged for 10 minutes at top speed in a clinical centrifuge. Red cell volumes were expressed as percentages of the total volume. Hemoglobin was measured by placing small volumes of sample in 2 cm^3 of 0.1% Na$_2$CO$_3$ and by reading optical densities at 545 mµ. Calcium in the fluid medium was precipitated as the oxalate and radioactivity in infinitely thick samples was counted with a gas flow counter.

In certain experiments, glucose (1.2 mM), adenosine (6 mM), or Nembutal (16 mg) was added to 100 cm^3 of stored blood. In other studies, acid-citrate-dextrose (ACD, Formula B, National Institutes of Health) was added to the blood. Saponin hemolysis was produced by adding 0.2 cm^3 of 4% saponin to the incubation mixture and hypotonic hemolysis was caused by adding water instead of salt solution to the isotope medium.

RESULTS

Calcium uptake by fresh and aged erythrocytes. Ca^{45} concentration in the fluid medium was lower when freshly drawn erythrocytes were incubated in KCl rather than in NaCl in 6 out of 7 experiments (table 1). Since RBC volumes were practically the same in either salt solution, these results indicate that more Ca^{45} dis-

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TABLE 1

Calcium uptake by fresh and stored erythrocytes

Date	Ca^{45} concentration in fluid medium, cpm/ml						Ca Uptake $\mu\mathrm{M/ml_{RBC}}$		pH		% Hemolysis		% RBC	
	Na			K										
	$(\mathrm{Ca}^{45})_{\mathrm{C}}$[1]	$(\mathrm{Ca}^{45})_0$[2]	$(\mathrm{Ca}^{45})_{30}$	$(\mathrm{Ca}^{45})_{\mathrm{C}}$[1]	$(\mathrm{Ca}^{45})_0$[2]	$(\mathrm{Ca}^{45})_{30}$	Na	K	Na	K	Na	K	Na	K
A. *Freshly drawn erythrocytes*														
10/7	694	964	874	699	985	715	0.019	0.055	7.32	7.38	2.9	1.8	28	29
11/2	568	789	675	560	800	670	0.029	0.033	7.42	7.42	1.9	2.7	28	30
11/9	585	835	660	585	813	570	0.042	0.061	7.48	7.72	—	—	30	28
1/4	515	696	650	500	667	548	0.013	0.036	7.43	7.57	0.6	0.5	26	25
1/11	420	545	562	405	664	378	−0.006	0.087	7.52	7.71	0.9	0.8	23	27
2/8	388	519	455	372	493	404	0.025	0.036	7.45	7.55	0.3	0.5	27	26
3/5	355	467	433	378	525	280	0.015	0.094	7.71	7.71	0.7	1.3	24	28
Mean	504	688	616	500	707	509	0.020	0.057	7.48	7.58	1.2	1.3	27	28
S.E.							±0.005	±0.009	±0.05	±0.05	±0.4	±0.4	±1	±1
							$P < 0.05$[3]							
B. *Erythrocytes stored for two days*														
10/9	695	914	715	702	861	270	0.044	0.130	7.30	7.37	4.7	2.7	24	28
11/4	590	792	725	598	832	241	0.017	0.144	—	—	—	—	26	26
11/11	490	653	575	507	658	287	0.024	0.114	7.42	7.45	4.7	5.1	25	23
1/6	490	681	595	425	574	131	0.026	0.156	7.43	7.50	1.0	1.3	28	26
1/13	440	579	528	400	533	59	0.020	0.180	7.59	7.59	0.9	2.9	24	25
Mean	541	724	628	526	692	198	0.026	0.145[4]	7.44	7.48	2.8	3.0	26	25
S.E.							±0.005	±0.011	±0.06	±0.05	±1.1	±0.8	±1	±1
							$P < 0.01$[3]							

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[1] The control tube, C, contained 1.5 ml of isotonic salt solution instead of 1.5 ml of red cell suspension.

[2] Time 0 values are determined by dividing Ca^{45} concentration in control tubes, $(\mathrm{Ca}^{45})_{\mathrm{C}}$, by the fluid volume.

[3] The differences between the means were evaluated by the paired *t* test (Batson, '56).

[4] Calcium uptake by stored cells suspended in KCl is significantly greater ($P < 0.01$) than the uptake by fresh cells suspended in the same medium.

ppeared when red cells were placed in ıe KCl medium. When cells from blood tored in a refrigerator for two days were tudied, there was even greater uptake of adioisotope by KCl-suspended cells but o significant effect of storage was ob- erved with cells incubated in NaCl. These hanges were essentially the same whether , 2, or 5-day-old cells were used. Nor vere any differences seen if glucose or denosine had been added to the stored lood.

With aged cells suspended in KCl, the nagnitude of the Ca^{45} decrease was greater han could be accounted for by equilibra- ion of the isotope in the cellular space. 'or example, in the experiment on day /6 (table 1), the Ca^{45} concentration in he medium was 574 cpm at time 0 and, f the cells had become completely perme- ble to the isotope, the expected count vould have been approximately 425 cpm, he value obtained in the control tube vhich contained salt solution instead of ed cell suspension. The observed value f 131 cpm indicates that Ca is bound to ell structures and that the increased up- ake during storage is due to an increase n the number of binding sites.

A comparison of Ca^{45} concentrations at imes 0 and 30 minutes with NaCl-sus- ended cells suggests that there is a small uptake of Ca^{45} in the presence of Na ion. Ca^{45} concentration at time 0, however, is a value calculated from the added Ca^{45} divided by the observed fluid volume. If the observed fluid volume were lower than the true volume by about 10%, there would have been essentially no difference between initial and final Ca^{45} concentrations. Calculations[2] indicate that the volume of fluid trapped with packed red cells may account for such a difference in volume so that the present experiments do not provide clear evidence of uptake in NaCl. However, Ca^{45} disappearance from the KCl medium cannot be explained in these terms since the trapped fluid volume was calculated to be equal to the observed erythrocyte volume in the case of fresh

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[2] An estimate of the trapped fluid volume may be obtained by subtracting the observed fluid volume from a calculated volume of distribution for Ca^{45} in the NaCl medium containing red cells, i.e., 2.5 cm³ × $(Ca^{45})_C/(Ca^{45})_{30}$, where the isotope concentrations are those in control and experimental tubes, respectively. It is assumed that Ca^{45} is not taken up in NaCl, so that the radioisotope may be used as an indicator of the volume of distribution. On this basis, an average of about 11% of the total fluid volume appears to be trapped with the sedimented red cells which may be compared with the value of 7% obtained by Kahn ('58) following centrifugation of whole human blood at greater forces and for longer periods.

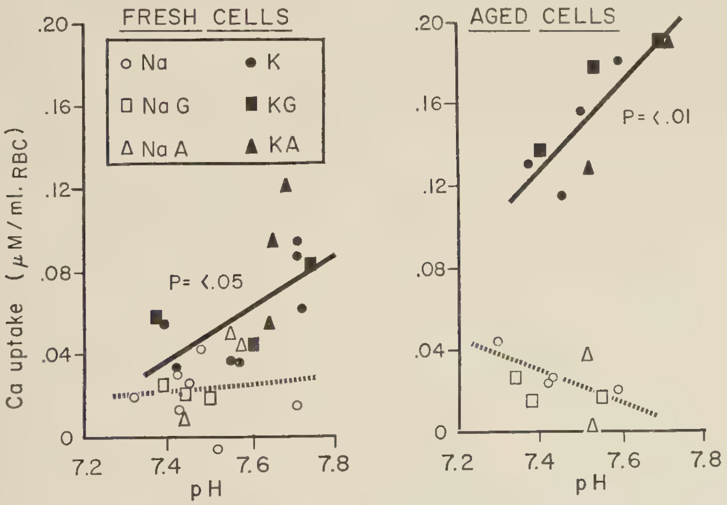

Fig. 1 Calcium uptake as a function of pH. Dog red blood cells were suspended in either isotonic NaCl (Na) or isotonic KCl (K). In certain experiments, glucose (G) or adenosine (A) was added to the blood before erythrocytes were separated, washed, and suspended in the salt medium.

cells and over three times the erythrocyte volume with aged cells.

Ca uptake has also been calculated in terms of μM/ml of RBC. The difference in total counts present in the medium at time 0 and at time 30 minutes was divided by the specific activity of the medium Ca at time 0 and by the original packed cell volume. The expression of results in these terms does permit a more convenient comparison of data from different experiments and an estimate of the order of magnitude involved. Thus, Ca uptake in NaCl was 0.02 and 0.03 μM/ml of RBC in fresh and stored cells, respectively, and corresponding values in KCl were 0.06 and 0.15. This calculation does not provide us with a true measure of rate since the time course of the Ca^{45} change as well as of the specific activity of precursor Ca are unknown.

pH and hemolysis. Calcium uptake in individual experiments has been plotted as a function of pH in figure 1. In different experiments, a pH range from 7.30 to 7.74 was observed which was due presumably to variation in individual blood samples as well as to the limited capacity of the added buffer to control pH. Regression lines were tested for significance by the analysis of variance (Batson, '56) and found to be meaningful for fresh and stored cells in KCl. It seems clear that the differences in Ca^{45} uptake in the two salt solutions could not be attributed to pH differences, especially with aged cells. Thus, the K regression line would not have been so widely separated from the Na line if the differences in Ca^{45} uptake were due solely to pH.[3]

Since hemolysis was slightly greater in KCl than in NaCl in most cases (tables 1, 2), our major observations could have been related to changes associated with hemolysis. As noted in table 2, fresh cells hemolyzed by saponin or by hypotonic media did not take up more Ca^{45} than unhemolyzed cells. This result appears to indicate also that the transfer of cellular material into the fluid medium is not related to our findings.

Influence of anesthetic, anticoagulant, and buffer. Since our principal observations could have depended upon the set of conditions originally chosen, additional studies were conducted in which defibrinated blood was obtained from etherized dogs and red cells were incubated in media buffered with imidazole. As shown in table 2, as the E, D, I experiments, the results were essentially the same as in the original series. However, it was noted that Ca uptake by aged cells in KCl was greater if cells were obtained from defibrinated instead of heparinized blood. In the presence of Nembutal, mean uptake appears to be slightly greater but the individual experiments did not show this difference consistently.

Blood was stored in acid-citrate-dextrose in a few experiments. Red cells stored in this manner apparently retain the characteristics of fresh erythrocytes since the marked increase in Ca uptake generally seen with stored cells was not observed. The ACD solution was probably not completely removed by two washings so that it is not altogether clear whether the acid pH or possibly the chelating action of citrate could have been primarily responsible. Nevertheless, the conditions of storage commonly employed in the storage of human blood seem to prevent Ca from being taken up in large amount by stored dog erythrocytes.

Species difference. Human and cat red blood cells were studied in the same manner as dog erythrocytes and it was found that neither the difference in uptake in KCl and in NaCl nor the storage effect could be demonstrated in these erythrocytes.

DISCUSSION

The disappearance of radiocalcium from the medium was found to be greater when dog erythrocytes were suspended in isotonic KCl rather than in isotonic NaCl. The difference was more clearly observed

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[3] In the majority of experiments, the pH was slightly greater in KCl solutions compared to NaCl solutions (tables 1, 2). This result, however, is not believed to be related to erythrocyte interactions because similar findings were obtained when NaCl and KCl solutions were read alternately in the absence of erythrocytes. This effect may have been due to differences in liquid junction potential at the calomel electrode brought about by differences in the concentration gradient of K ion or to the presence of Na ion at the interphase during a portion of the readings. pH differences between individual blood samples, however, are considered to be reliable because of the relatively small variability in the readings with a given solution.

TABLE 2

Calcium uptake by dog erythrocytes under various experimental conditions[1]

Condition	Days of storage	pH		% Hemolysis		% RBC		Ca uptake $\mu M/ml_{RBC}$		No. of expts.
		Na	K	Na	K	Na	K	Na	K	
Control	0	7.48	7.58	1.2	1.3	27	28	0.020	0.057	7
	2	7.44	7.48	2.8	3.0	26	25	0.026	0.145	5
Glucose	0	7.44	7.57	1.3	1.1	30	23	0.021	0.061	3
	2	7.42	7.54	1.7	3.4	24	24	0.026	0.169	3
Adenosine	0	7.52	7.66	0.6	0.6	25	26	0.034	0.090	3
	2	7.52	7.61	1.4	4.5	23	23	0.017	0.171	3
Saponin hemolysis	0	7.58	7.63	17.7	19.5	19	14	0.037	0.046	2
Hypotonic hemolysis	0	7.38	7.45	6.4	10.3	33	28	0.039	0.049	4
Acid-citrate-dextrose	0	6.77	7.10	1.1	0.9	29	25	0.038	0.045	1
	2	6.72	6.98	2.3	1.7	31	28	0.035	0.039	2
	4	6.78	6.80	1.7	1.5	27	28	0.031	0.037	2
E,D,I	0	7.37	7.47	0.8	1.1	24	23	0.021	0.047	3
	1	7.34	7.47	1.6	3.1	26	26	0.033	0.164	3
E,H,I	0	7.25	7.49	1.2	1.3	28	24	0.031	0.035	3
	1	7.27	7.40	1.7	1.7	30	27	0.038	0.084	3
E,D,I,N	0	7.38	7.57	0.6	1.1	30	27	0.027	0.055	2
	1	7.46	7.55	2.5	3.6	31	28	0.047	0.174	2
E,H,I,N	0	7.26	7.49	1.2	1.0	34	32	0.036	0.052	2
	1	7.31	7.45	2.6	2.9	34	33	0.047	0.103	2
Cat RBC	0	7.47	7.56	0.2	0.2	29	27	0.034	0.041	2
	2	7.55	7.46	0.5	0.4	27	28	0.037	0.035	2
Human RBC	0	7.57	7.57	0.3	0.3	22	25	0.014	0.010	2
	1	7.53	7.56	0.3	0.2	24	24	0.008	0.014	2
	5	7.51	7.53	0.6	0.4	23	22	0.006	0.001	1

[1] The following abbreviations are used: E, etherized animal; D, defibrinated blood; H, heparinized blood; I, imidazole buffer; and N, nembutal added to stored blood.

when two-day-old cells were used. The simplest explanation for this result may be that in NaCl, a competition for binding sites takes places between Ca and Na ions due to the similarity in ionic radii, as proposed by Mullins ('56). In KCl, a comparable interaction apparently does not take place due to the difference in ion size.

The greater uptake of radiocalcium in KCl by aged cells indicates that some change takes place during cold storage. In contrast to fresh cells, stored human erythrocytes have been found to lose more ipid and lipoprotein presumably from the cell membrane when washed several times with 0.16 M NaCl (Lovelock, '56). It seems possible that a similar loss of "membrane material" may occur when dog red blood cells are washed with 0.16 M KCl and that the greater loss of this maerial following storage could account for the greater Ca uptake by the uncovering of additional binding sites. Even under these conditions, Ca^{45} uptake was less, if it occurred at all, in NaCl.

The storage effect appears to be independent of substrate lack since no difference was seen when cells had been stored in glucose or in adenosine. Glucose utilization has been observed in dog red blood cells (Sheppard et al., '51) and both substrates prolong the viability of stored human erythrocytes (Mollison and Young, '42; Gabrio et al., '55). Our procedures give no indication as to whether net uptake of Ca may have occurred but Armstrong and Singer ('53) have reported decreases in plasma Ca of as much as 23% in cold-stored, heparinized dog blood.

In our experiments, acid-citrate-dextrose prevented the appearance of the storage effect. Since one feature of an aging cell appears to be an increase in Ca binding capacity (Heilbrunn, '56), the mechanism whereby ACD delays senescence in erythrocytes may be concerned with the inhibition of Ca uptake. An alternate possibility is the proposal of Lovelock ('56) that ACD exerts a poor solvent action towards stroma lipoprotein. However, our ACD experiments were performed with dog blood and it should be emphasized that a storage effect was not seen with human blood. On the other hand, Kahn ('58) has observed that more radiocalcium disappears from plasma when stored, in contrast to fresh, human blood was incubated for 2–6 hours.

Ca uptake by cells suspended in KCl appeared to be dependent upon pH. The greater uptake at alkaline pH may have been due to the increase in negative sites on cell protein as pH is raised. Ca binding by proteins has been observed and, in one case (Chanutin et al., '42), the binding of Ca was found to decrease with increasing NaCl concentration although ionic strength was also varied. In the present study, the increased availability of anionic sites with increased pH did not affect Ca uptake in NaCl solutions indicating that the affinity of Na for binding sites is apparently greater even under these conditions.

SUMMARY

1. The disappearance of Ca^{45} from the medium was greater when washed dog erythrocytes were suspended in isotonic KCl rather than in isotonic NaCl. Cells stored in a refrigerator for 24 hours or more took up even greater quantities of Ca^{45} when incubated in KCl but cells suspended in NaCl did not show any difference from fresh cells. This result is consistent with the view that competion takes place between Ca and Na ions for binding sites as a consequence of the similarity in ionic radii.

2. Acid-citrate-dextrose and, to a certai extent, heparin appeared to delay the in creased uptake by stored cells. Addition of glucose, adenosine, or Nembutal t stored blood had no effect. Fresh cell hemolyzed by saponin or by hypotoni media took up no more Ca than unhemo lyzed fresh cells.

3. Calcium uptake in KCl was depend ent upon pH, greater amounts being taken up at alkaline pH.

4. In contrast to dog red cells, human and cat erythrocytes did not show differ ences in uptake in NaCl and in KCl, befor or after storage.

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Asphyxial Changes in the Cerebellar Cortex[1]

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The changes produced in the cerebral cortex by asphyxiation and by spreading depression are qualitatively quite similar. Both cause surface negativity of the cortex (Leão, '47, '51) and an increase in cortical mpedance (Leão and Ferreira, '53; Freygang and Landau, '55; Van Harreveld and Ochs, '56, '57). Furthermore both result in a transport of water (Van Harreveld, '57, '58) and electrolytes (Van Harreveld and Schadé, '59) from an extraneuronal space into apical dendrites. These similarities suggested a basic identity of the processes underlying the asphyxial changes and spreading depression (Leão, '51; Van Harreveld and Stamm, 53; Van Harreveld, '58; Van Harreveld and Schadé, '59; Bureš and Burešová, '60). Spreading depression has not been described in the cerebellum and attempts in this laboratory to elicit this phenomenon n the cerebellar cortex have met with failure. Leão had a similar experience (personal communication). In view of the inability of the cerebellar cortex to elaborate spreading depression it was of nterest to investigate the effect of asphyxiation on the cerebellum and to look in particular for impedance changes and the transport of water and electrolytes in its cortex.

METHODS

The physiological experiments were performed on rabbits. The cerebellar cortex was exposed in ether narcosis, but the measurements and recordings were carried out under Intocostrin (E. R. Squibb and Sons, New York) immobilization. Potentials were led off between an electrode placed on the vermis and an indifferent one on the ear. The electrodes for the mpedance measurements were silver discs 1½ to 2 mm in diameter which were placed 3–4 mm apart on the same folia of the vermis. In attempts to elicit spreading depression a third, stimulating electrode was placed on these same folia. The impedance bridge described previously was used (Van Harreveld and Ochs, '56). The measuring current was a 1000-cycle sinusoidal current attenuated below the threshold of stimulation. Asphyxiation was caused by severing the abdominal aorta.

The water and electrolyte transport was investigated in rabbits and rats. The histochemical technique used, which shows either the location of chloride alone, or in addition to this, phosphate, carbonate and an unidentified (probably organic) anion, has been described in detail (Van Harreveld and Potter, '61). The treatment started with freezing the exposed vermis by pouring isopentane cooled to its fusion point into a cup of skin made by sewing the edges of the wound exposing the cerebellum to a steel ring. The frozen tissue was then kept in 90% alcohol saturated with silver nitrate (about 2.5%) at a temperature of −25°C. During the ensuing substitution fixation silver ions diffused into the tissue precipitating the anions mentioned above. After one week the tissue was washed for 4 to 5 hours in a repeatedly changed molar sodium nitrate solution at 0°C, then for an additional 12–18 hours in the same solution at room temperature. The tissue blocks were dehydrated, embedded in paraffin and sectioned (10 μ). To observe the position of all the electrolytes precipitated by silver ions the sections were mounted without further treatment and exposed to sunlight. To study the location of the chloride alone, the preparations were deparaffinized and passed for a few minutes through a 1% nitric acid (volume) solution which dissolved all silver salts but the

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chloride. After washing, the sections were mounted and exposed to light.

RESULTS

Physiological experiments

Spreading depression. In agreement with previous attempts no spreading depression was observed in the cerebellar cortex even when stimulating with strong direct currents or with concentrated potassium chloride solutions. Both are very effective in producing spreading depression in the cerebral cortex of the rabbit. Since the impedance change is a reliable sign of spreading depression this has been looked for mostly. In a few experiments an unsuccessful search for slow potential changes was made.

Some procedures have been described which enhance the tendency of the cerebral cortex to react with spreading depression on adequate stimulation. Effective in this respect are dehydration (Marshall, '50), long exposure of the cortex (Marshall and Essig, '51), cooling (Marshall, Essig and Dubroff, '51), treatment of the cortex with isotonic sucrose solutions (Essig and Marshall, '50) or with solutions containing abnormally high potassium concentrations (Marshall, Essig, and Witkin, '51). With these procedures spreading depression can be produced in the retrospleneal area of the rabbit in which it cannot normally be elicited (Van Harreveld and Bogen, '56). It is possible that application of any of these treatments would also enable the cerebellar cortex to respond with spreading depression. This has not been tried in the present series of experiments, however.

Asphyxial changes. An impedance increase develops consistently in the cerebral cortex after asphyxiation. For the first minutes after circulatory arrest the impedance tends to rise slowly and gradually. After about three minutes, however, a sudden and large impedance increase develops during which, in the course of about two minutes, 30% or more of the conductivity of the cerebral cortex is lost (Van Harreveld and Ochs, '56). Although small potential variations may occur during the first minutes of asphyxiation (Van Harreveld and Stamm, '53), a pronounced surface negativity of the cerebral cortex develops at the start of the sudden impedance rise (Van Harreveld and Ochs, '56).

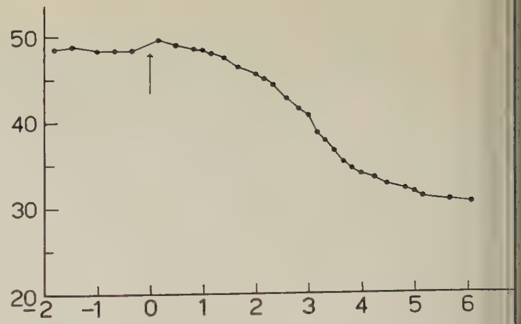

Fig. 1 Loss in conductivity of cerebellar cortex during asphyxiation. The conductivity, expressed in mhos × 10^5, is plotted on the ordinate, time in minutes on the abcsissa. At the arrow the circulation is arrested.

The impedance of the cerebellar cortex also increased after asphyxiation. This increase was of the same magnitude as in the cerebral cortex; its time course was somewhat different, however. In the cerebellar cortex the impedance rose more gradually. Figure 1 shows the changes in asphyxial conductivity (the reciprocal of the impedance expressed in mhos × 10^5) of the cerebellum after circulatory arrest. Although there is an acceleration of the loss in conductivity two to three minutes after cutting the aorta, the sudden drop which is so typical for the cerebral cortex is lacking.

As in the cerebral cortex a surface negativity developed in the cerebellum after asphyxiation. This potential change started from one to three minutes after cutting the aorta, and was of considerable magnitude (15 to 20 mv) at its maximum. It sometimes consisted of several waves which could represent the asphyxial potentials picked up by the relatively large leading off electrode from two or three folia in which the potential developed with a slightly different time course.

Histological findings

The silver-substitution method was applied to cerebellar cortex frozen during normal oxygenation or a few seconds after circulatory arrest (controls), and after the asphyxial impedance increase had taken ace (experimental preparations). In bbits the vermis was exposed and a in cup prepared in ether narcosis then e preparation was immobilized with In- costrin. In control rabbits the cerebel- m was frozen while the circulation was tact and after it has been ascertained at the impedance was within the range r normal oxygenated cerebellar cortex. the experimental rabbits the vermis was ozen after the asphyxial impedance in- ease had taken place and the rate of ange was falling off, usually 6–7 min- tes after cutting the aorta.

In rats the vermis was exposed in ure- ane narcosis. In all preparations the rtex was left exposed for 15–20 min- tes, but care was taken to keep it moist. the controls the head was cut off and ansferred immediately to cold isopen- ne. In the experimental rats a period of minutes was interposed between severing e head and freezing to allow the develop- ent of the asphyxial changes.

Figure 2A is a photomicrograph of an id treated preparation which shows the stribution of chloride in rat cerebellar rtex frozen before, and figure 2B of a milar preparation frozen after the as- yxial changes had taken place. As was und in the cerebral cortex the chloride as rather uniformly distributed in the ntrol preparation. In the asphyxiated rebellar cortex a transport of chloride d taken place into certain cellular ele- ents which are darker whereas the tissue between them is lighter than in the ntrol preparation. Some of the struc- res into which chloride had moved could readily identified as dendrites of Pur- nje cells. Since the cortex had been t at right angle with the folia of the rmis the dendritic arborizations of these lls are in the plane of the section. It as especially in the main dendritic unc(s) and the first branches that chlo- de had accumulated (fig. 2B, C). The ore superficial part of the arborization hich consists of smaller branches was t visible in these preparations. No con- stent transport of chloride into the peri- rya of the Purkinje cells was observed cept at the origin of the dendritic unc(s) (fig. 2B).

There are, however, other structures in which chloride is concentrated. They consist of relatively thick fibers which in favorable preparations (fig. 2D) can be seen to begin close to the granular layer and run all the way to the surface of the cortex. Some of these fibers branch but the great majority run undivided and straight through the entire molecular layer. Although the axons of the granular cells take a similar course, they divided in their two branches at all levels of the molecular layer. Furthermore these axons are very thin whereas the fibers taking up the electrolytes have a considerable diameter. Also dendrites of the Golgi cells or grand cellules étoilées (Cajal, '55), the perikarya of which are situated in the granular layer, have a course in the plexiform layer somewhat similar to that of the fibers which take up chloride. The dendrites of the Golgi cells branch rather profusely, however, and often do not reach the cerebellar surface. Furthermore close to the granular layer these dendrites often run parallel with the surface of that layer, which is never observed of the fibers taking up chloride after asphyxiation. These latter fibers can thus neither be readily identified with the axons of the granular cells, nor with the dendrites of the Golgi cells. However, these structures do resemble strikingly the fibers of the epithelial cells of Golgi (Cajal, '55) also called the "Stüzzellen" of Bergmann (Jansen and Brodal, '58), a glial component of the cerebellar cortex. Figure 2E shows these elements in rabbit cerebellum treated with a glia stain. The cells of origin of the fibers are relatively small and adjoin the molecular layer. The fibers which arise from these cells run with a minimum of branching straight through the molecular layer to the surface where they form end feet. The end feet form together the limiting membrane of the cerebellar cortex. A comparison of the fibers stained with the silver-substitution and with the gold sublimate methods as shown in figure 2D and E, demonstrates the similarity of their course.

No transport of chloride into the cell bodies of Bergmann's "Stützzellen" could be demonstrated. An inspection of the granular layer in preparations frozen before and after the asphyxial changes had

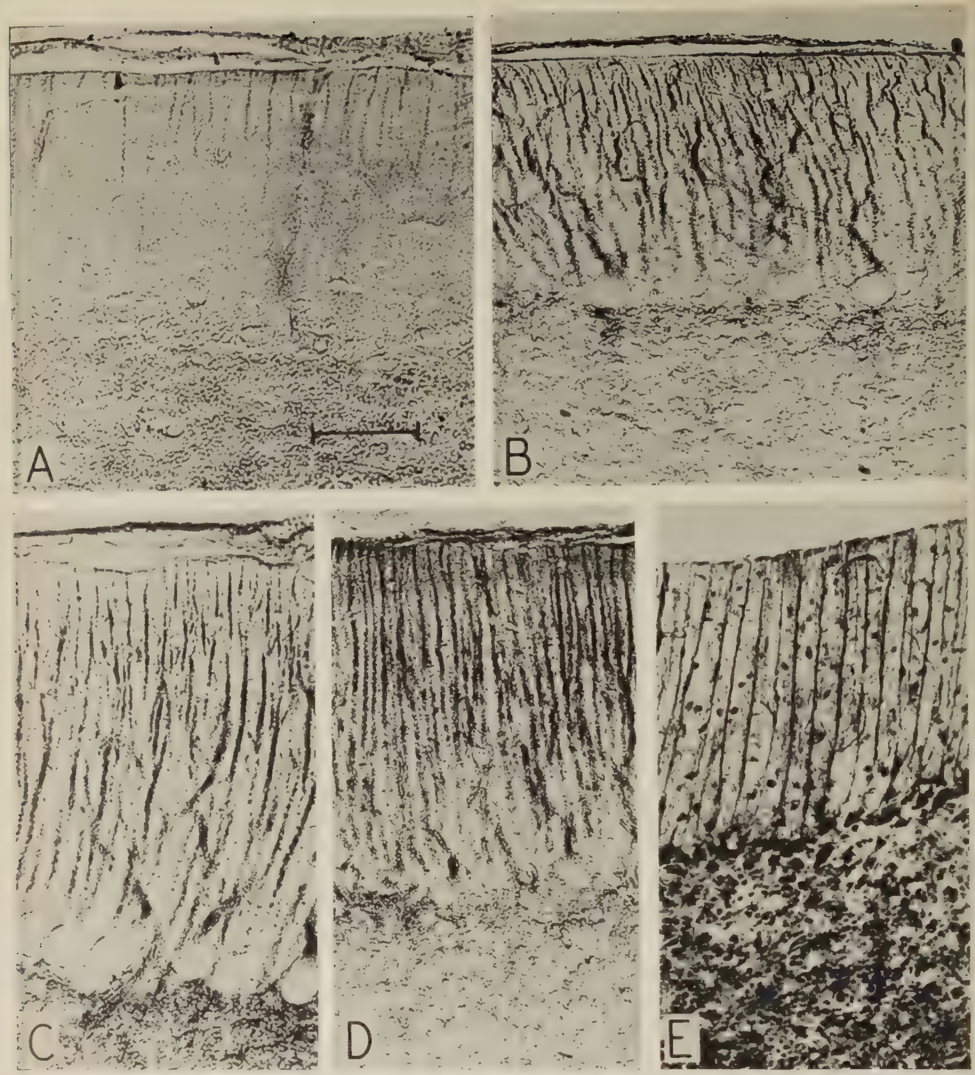

Fig. 2 Photomicrographs of cerebellar cortex treated with the silver-substitution method. The preparations shown in A and B are of rat cerebellum which had been treated with nitric acid. A is cortex frozen before, B after the asphyxial impedance increase had occurred. The photomicrographs C and D are asphyxiated cerebellar cortex of the rabbit treated with neutral solutions. To enhance the contrast a green filter was used in making the pictures A, B, C and D. E is rabbit cerebellum stained with Cajal's gold-sublimate method. The magnification of all the photomicrographs is the same. The horizontal calibration line in A indicates 100 μ.

taken place did not reveal consistent differences in chloride distribution.

As mentioned above the chloride was rather uniformly distributed in control preparations of the rat frozen a few seconds after circulatory arrest. However, even in these preparations the outlines of the large dendrites of the Purkinje cells and of the fibers of Bergmann could b faintly seen. In the control preparatio shown in figure 2A some fibers of Bergmann are visible in the top of the plexifor layer.

Preparations of rat cortex treated wit neutral solutions and thus showing besides chloride the other ions precipitate by silver nitrate (carbonate, phosphate and an unidentified, organic anion) were somewhat darker than the acid treated preparations which show chloride alone. The distribution of these electrolytes in control and experimental preparations was very similar to that of acid treated preparations, however.

The cerebellar cortex of rabbits showed the same rather uniform distribution of electrolytes in the oxygenated controls and a concentration of these ions in the large dendrites of the Purkinje cells and in the fibers of Bergmann in the experimental preparation as described above for the rat. Figure 2C and D which are photographs of preparations of asphyxiated cerebellar cortex of the rabbit treated with neutral solutions demonstrate the electrolyte transport in this species.

In most preparations the electrolyte transport had taken place both into the fibers of Bergmann and into the dendrites of the Purkinje cells. In such preparations electrolytes had moved mainly into the dendrites in the bottom of the plexiform layer, whereas the transport had taken place only into the fibers of Bergmann in the top of that layer (fig. 2B and C). In some cerebellums, however, the electrolytes had moved almost exclusively into the Bergmann fibers (fig. 2D) which then can be followed easily from the granular layer to the surface of the cerebellums.

Ratio between Bergmann's fibers and Purkinje cells in the cerebellar cortex of rats and rabbits. On the basis of their anatomical features the fibers in the molecular layer of the cerebellar cortex which take up electrolytes after circulatory arrest were identified as the fibers of Bergmann's "Stützzellen." It was attempted to support this conclusion by determining the ratios between the Purkinje cells and these fibers in preparations stained with a glia stain in which the fibers of Bergmann and their cells of origin can be identified with certainty, and in preparations treated with the silver-substitution method. This ratio rather than the number of fibers in a known volume of cerebellar cortex was chosen because of the great differences in the treatment of the tissues which may cause different shrinkage. For the glia stain Cajal's gold-sublimate method (Conn, Darrow and Emmel, '60) was used (fig. 2E). The Purkinje cells were sufficiently stained in these preparations to be easily recognizable. The staining of asphyxiated cerebellar cortex with the silver-substitution method makes the fibers readily visible. These preparations were counterstained with methylene blue to facilitate the recognition of the Purkinje cells. In both kinds of preparations all the Purkinje cells and all the fibers were counted in well stained relatively straight stretches of cortex. The curved cortex at top and bottom of the folia was avoided because of the differences in the density of Purkinje cells described there. In table 1 the results of these cell and fiber counts are given. Four rat and 4 rabbit cerebellums were used for each of the staining methods. One to 4 folia were chosen of each cerebellum which were sufficiently long to make the counting of a reasonable number of cells and fibers possible. The table shows the number of cells and fibers and their ratio in each of these folia. In the preparations of rats treated with the silver-substitution method a total of 171 cells and 691 fibers were counted which gives a ratio of 4.0. In the preparations stained with the Cajal method these numbers were 199 and 870, which gives a ratio of 4.4. The means of the ratios in individual folia with the two staining methods were 4.1 and 4.4 respectively, both with a standard error of 0.1. The difference between these two mean ratios was statistically not significant ($t = 2.0$).

In the rabbit 147 cells and 935 fibers were counted in the preparations treated with the silver-substitution method, which gives a ratio of 6.4. In the cerebellums stained with the gold-sublimate method these figures were 185 and 1086 with a ratio of 5.9. The means of the ratios in individual folia with the two methods of staining were 6.4 and 5.8 with standard errors of 0.3 and 0.2 respectively. Also the difference between these mean ratios was not significant ($t = 1.7$).

The similarity of the ratios indicates that the number of the fibers stained with the silver-substitution method and with a recognized glia stain is the same in a given volume of cerebellar cortical tissue. This strongly supports the identification of

TABLE 1

Ratio between Bergmann's fibers and Purkinje cells in the cerebellar cortex of rats and rabbits

Silver substitution method				Cajal's gold-sublimate method			
Cerebellum	Cells	Fibers	Ratio	Cerebellum	Cells	Fibers	Ratio
Rat 1	17	61	3.6	Rat 5	25	102	4.0
	12	54	4.5		32	133	4.2
Rat 2	22	97	4.4	Rat 6	25	112	4.5
	20	85	4.3		24	103	4.3
	27	107	4.0				
Rat 3	22	91	4.1	Rat 7	19	86	4.5
	13	52	4.0		26	106	4.0
Rat 4	38	144	3.8	Rat 8	23	109	4.7
					25	119	4.8
Means	171	691	4.1		199	870	4.4
Rabbit 1	12	87	7.2	Rabbit 5	17	82	4.8
	11	78	7.1		17	89	5.2
	21	128	6.1		12	70	5.8
					16	87	5.4
Rabbit 2	16	111	7.0	Rabbit 6	25	156	6.2
	12	54	4.5		21	133	6.3
	16	115	7.2				
Rabbit 3	16	93	5.8	Rabbit 7	15	106	7.0
	14	85	6.1		21	105	5.0
Rabbit 4	18	109	6.1	Rabbit 8	26	162	6.2
	11	75	6.8		15	96	6.4
Means	147	935	6.4		185	1086	5.8

the fibers which take up electrolytes after circulatory arrest as Bergmann's fibers.

Changes in the diameter of Bergmann's fibers after circulatory arrest. The asphyxial transport of electrolytes into the apical dendrites of the cerebral cortex is accompanied by a marked swelling due to a simultaneous transport of water to maintain osmotic equilibrium (Van Harreveld, '57). A similar swelling can be expected to occur in the fibers of Bergmann after circulatory arrest. The diameters of these fibers were measured in preparations frozen before and after the asphyxial electrolyte transport had taken place. This did not pose any difficulties in asphyxiated cerebellums where the fibers were well stained with the silver-substitution method. In the control preparations in which the fibers were much more faint this was less easy but still possible, especially when the preparations had been treated with neutral solutions. The fiber diameters were determined in a plane midway between the surface of the cerebellar cortex and the granular layer. The measurements were made under oil immersion, using a “Zeiss” camera lucida. The apparent diameters of the fibers were marked on the paper acting as a screen of the camera lucida, and were later measured to the nearest 0.1 of a mm. These figures will be used in the present paper Since the magnification of the optical system was about 1400 ×, multiplication with 0.72 gives the diameter in μ. The above method is the same as that employed for the measurement of apical dendrites in the cerebral cortex (Van Harreveld, '57).

Table 2 shows the diameters of Bergmann's fibers of rats. In 8 animals the cerebellum was frozen before the asphyxial changes could take place and in 8 preparations a period of 8 minutes was interposed between circulatory arrest and freezing. In each cerebellum all the fibers (about 100) in a well stained, straight stretch of cortex were measured. The mean diameters in the 8 control cortices varied between 2.6 and 3.3 mm (1.9 and

TABLE 2

Diameters of Bergmann's fibers in rats expressed in an arbitrary unit of length

No.	Frozen before asphyxial changes	No.	Frozen 8 min. after circulatory arrest
1	3.0±0.05	9	4.9±0.09
2	2.6±0.05	10	5.7±0.12
3	2.8±0.05	11	5.3±0.13
4	2.6±0.04	12	4.9±0.08
5	2.6±0.05	13	5.5±0.11
6	3.0±0.05	14	5.2±0.11
7	3.3±0.07	15	5.2±0.12
8	3.0±0.06	16	4.9±0.09
Means	2.9±0.02		5.2±0.04

2.4 μ), the standard errors were small (0.04 to 0.07 mm). The mean diameters after 8 minutes asphyxiation were considerably larger, ranging between 4.9 and 5.7 mm (3.5 and 4.1 μ) with larger standard errors (0.08 to 0.13 mm). The mean diameter of the 800 fibers in the control preparations was 2.9 mm (2.1 μ) ± 0.02 mm and in the asphyxiated cerebellar cortices 5.2 mm (3.7 μ) ± 0.04 mm. This difference is highly significant (t = 55). Figure 3B shows histograms based on these measurements. The diameters in the control preparations show little variability whereas the diameters of the experimental cortices vary considerably. Since these are the same populations of fibers the swelling was apparently not uniform.

Table 3 shows a similar series of experiments with rabbits. The diameters of Bergmann's fibers in 6 control experiments varied between 2.8 and 3.1 mm (2.0 and 2.2 μ). In 6 asphyxiated cerebellums the diameters were between 4.7 and 6.0 mm (3.4 and 4.3 μ). The mean of all 600 control measurements was 3.0 mm (2.2 μ) ± 0.02 mm. The mean diameter in all the experimental cerebellar cortices was 5.2 mm (3.7 μ) ± 0.04 mm. This difference is again highly significant (t = 46). Figure 3A shows histograms of all fiber diameters measured in control and experimental rabbit cerebellums. As in the rat the variability in the experimental preparations was greater than in the controls. The increase in diameter which is about the same in rats as in rabbits is very considerable (70 to 80%). This corresponds to a volume increase of Bergmann's fibers of about 300%.

Horstmann and Meves ('59) pointed out that differences in the intensity of silver impregnations in preparations treated with one of the routine methods for the nervous system could account for differences in diameter of fibers since silver is precipitated on the surface of the stained structures. In the present instance there is a considerable difference in the staining of Bergmann's fibers in the control and experimental preparations. However, the method employed shows the presence of electrolytes and is not based on the precipitation of silver compounds on surfaces. The increase in diameter observed, must therefore be considered as a true swelling of the fibers after circulatory arrest.

DISCUSSION

The asphyxial changes in the cerebellar cortex described above are similar to those in the cerebral cortex. In both structures the impedance increases considerably, and a transport of electrolytes and water into cellular elements takes place. There are differences, however. In the cerebral cor-

TABLE 3

Diameters of Bergmann's fibers in rabbits expressed in an arbitrary unit of length

No.	Oxygenated cerebellum	No.	Asphyxiated cerebellum	Interval between circulatory arrest and freezing
1	3.0±0.05	7	5.0±0.09	6′ 10″
2	2.8±0.04	8	6.0±0.09	6′ 30″
3	2.9±0.04	9	5.5±0.10	7′ 30″
4	3.1±0.05	10	4.8±0.10	6′ —
5	2.9±0.04	11	4.7±0.08	6′ 40″
6	3.1±0.07	12	4.9±0.11	6′ 50″
Means	3.0±0.02		5.2±0.04	

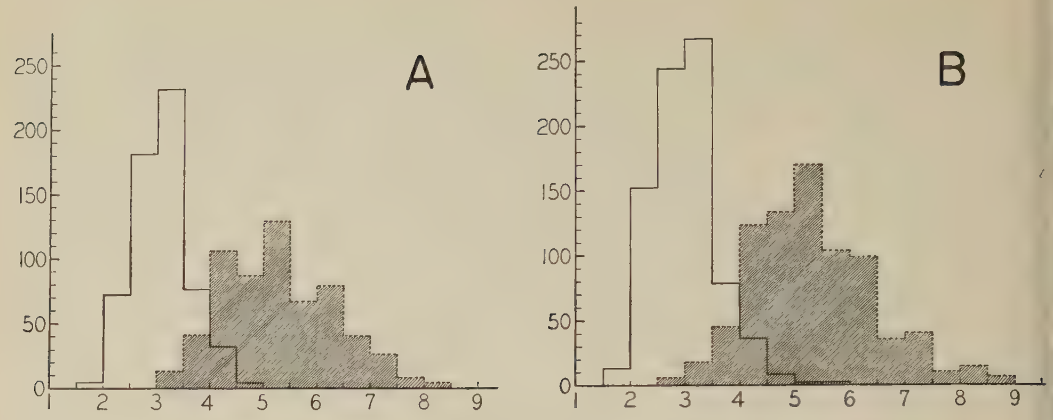

Fig. 3 Histograms of the diameters of Bergmann's fibers. On the ordinate are plotted the number of fibers, on the abscissa the diameter in an arbitrary unit of length. A shows two histograms, each based on 600 measurements in the rabbit. The non-hatched histogram of cerebellums frozen while the circulation was intact; the hatched histograms of cerebellums frozen after the circulation had been arrested for 6–7 minutes. In B two similar histograms each based on 800 measurements in rats are shown. The non-hatched histogram is of cerebellums frozen a few seconds after cutting off the head. A period of 8 minutes was interposed between severing the neck and freezing of the cerebellums used for the hatched histogram.

tex the electrolytes moved primarily into the apical dendrites. There was also some transport into the pyramidal cells, but no evidence of an electrolyte movement into glial elements was found. In the cerebellar cortex electrolytes not only moved into dendrites of the Purkinje cells but in addition to this into Bergmann's fibers, an element of the cerebellar cortex which is generally considered to be of glial nature. In some preparations the latter was even the most pronounced electrolyte movement.

As mentioned above the similarity between the features of spreading depression and of the asphyxial changes in the cerebral cortex is so great, that the processes underlying these phenomena are assumed to be identical (Leão, '51; Van Harreveld and Stamm, '53; Van Harreveld, '58; Van Harreveld and Schadé, '59; Bureš and Burešová, '60). It has been suggested that the basic mechanism underlying the features both of spreading depression and of the asphyxial changes is an increase of the ion permeability of the cell membrane (Van Harreveld and Ochs, '56, '57; Van Harreveld and Schadé, '59, '60). A similar membrane change can be expected to result in the asphyxial electrolyte movement into the nervous and glial elements of the cerebellum. Since the asphyxial potential is in all probability the result of these membrane changes it seems likely that both neural and glial elements are responsible for the surface negativity in the cerebellum after circulatory arrest.

It is of interest that the asphyxial electrolyte transport is observed in certain structures and not in other, similar ones. For instance electrolytes move regularly into the apical dendrites of pyramidal cells in the cerebral cortex but not into the basal dendrites. An explanation for this observation can be sought in the competition of various structures for a limited amount of chloride in the cortex (Van Harreveld and Schadé, '59). If during asphyxiation the membrane changes responsible for the ion transport start in the apical dendrites, then the available chloride may have been exhausted before such changes occur in the basal dendrites. Such reasoning can be used to explain the observed variations in the asphyxial electrolyte transport in the cerebellum. In some cerebellums the transport occurred exclusively into the fibers of Bergmann, in others the electrolytes moved also into the dendrites of Purkinje cells. According to the above view the membrane changes would in some preparations have occurred first in the Bergmann fibers, in others they would have developed simultaneously in these fibers and in the dendrites of the Purkinje cells.

In the cerebral cortex the asphyxial impedance increase develops suddenly after a latency of about three minutes, accompanied by the asphyxial potential (Van Harreveld and Ochs, '56). In the cerebellum the impedance rose more slowly and gradually. The latter feature may be related to the inability of the cerebellar cortex to elaborate spreading depression. Leão ('47, '51) observed during asphyxiation of the cerebral cortex the development of foci of surface negativity with different latencies. From such foci negativity may spread by a process identical or similar to spreading depression, engulfing the entire cortex in a short time. Such a concept which is supported by Bureš and Burešová's ('60) finding that asphyxiation activates latent foci of spreading depression can account for the sudden impedance increase of the cerebral cortex. In the cerebellum asphyxial membrane changes starting in certain foci would not be able to spread since in this part of the nervous system the conditions for phenomena like spreading depression are not favorable. As asphyxiation proceeds membrane changes may develop at other spots until finally the entire cerebellar cortex is involved. Such a concept agrees with the more gradual development of the impedance rise in the cerebellum.

The observation that not only nervous elements but also glia cells can participate in the asphyxial water and electrolyte transport enhances the potential magnitude of intracortical shifts of material. Estimates of extracellular space based on the study of material fixed for light- or electronmicroscopy in which such shifts can be expected to have taken place should therefore be regarded with caution.

SUMMARY

As in the cerebral cortex asphyxiation produces surface negativity and an impedance increase in the cerebellar cortex. A histochemical method indicating the presence of choloride alone, or in combination with phosphate, carbonate and an unidentified (probably organic) anion was applied to cerebellar cortex of rabbits and rats either before, or after the asphyxial impedance increase had taken place. A rather uniform distribution of these electrolytes was observed in the plexiform layer of cortices in which the asphyxial changes had not taken place. In asphyxiated cerebellums the electrolytes were concentrated in the main dendrites of the Purkinje cells and in structures which by their anatomical features were identified as the fibers of Bergmann's "Stützzellen" (epithelial cells of Golgi), a glial component of the cerebellar cortex. This identification was supported by the demonstration that in preparations treated with the histochemical method the ratio between these fibers and the Purkinje cells, and in cerebellar cortex treated with a glia stain the ratio between the fibers of Bergmann and the cells of Purkinje are similar. A comparison of the diameter of the fibers of Bergmann in asphyxiated and non-asphyxiated cerebellar cortex indicates that a 70–80% increase in thickness takes place during the asphyxial impedance rise. This corresponds to an increase in the volume of these structures of about 300%.

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Osmotic Hemolysis by a Gradual Decrease in the Ionic Strength of the Surrounding Medium

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A method has been previously described for the preparation of RBC ghosts by gradually decreasing the salt concentration of the surrounding medium in which the erythrocytes are suspended (Danon et al., '56). This was achieved by introducing the erythrocyte suspension (in buffered isotonic NaCl solution) into dialysis tubing and placing it into a flask containing a hypotonic solution. This procedure permits a gradual decrease in the salt concentration, thus producing "ghosts" in a very high yield and without, at the same time, causing tears or craters in the membranes. Further study of this technique (Klibansky, '59) led to a better understanding of the process and differences were found in the reaction of the membrane to the decreasing salt concentration according to whether the decrease was slow (by dialysis) or "drastic" (by throwing the erythrocytes directly into hypotonic solution). This study was restricted to observations on a population of erythrocytes. Still no technique was available for the continued microscopic observation of one or a specific group of erythrocytes in a slow process of deformation and swelling leading ultimately to the release of hemoglobin through the membrane. The escape of the hemoglobin might be due either to a tear in the membrane, or by the membrane becoming permeable over its entire surface while the salt concentration in the surrounding medium is decreasing and the pressure inside the cell is increasing (Ponder, '48; Parkart and Ballentine, '52; Bessis, '54).

Hemolysis, caused by various hemolyzing agents, other than osmotic, has been followed by microcinematography through the interference microscope by Marsden ('56). A high speed photography at 540 mμ is mentioned by Parpart ('52) with no indication of cinematography.

In the present study, a new method was employed in order to follow the deformation of 0.2 ml of diluted suspension of normal human bidiscoid erythrocytes under the phase contrast microscope. This was achieved by using a mixed ion exchanger that takes out of the solution Na and Cl ions and releases into the suspending medium H and OH ions. This permits reduction of the ionic strength while maintaining the pH relatively constant. Under these conditions it was possible to follow the deformation caused by the increasing internal dilution (decreasing ionic strength of the surrounding medium), until they lost their hemoglobin and became ghosts.

MATERIAL AND METHODS

Two drops of normal human blood were taken from a finger-tip after taking special precautions for cleanliness according to Trotter ('56). For this purpose the finger-tip was wiped with ether and allowed to dry before being punctured with the needle. The two drops were introduced directly into a test-tube containing 2 ml of isotonic buffered (pH 7.4) saline.

The test-tube used to receive the saline and the blood was flamed on a Bunsen burner before use, until the glass was soft. The tube was rotated while being flamed, when deformation of the tube began, it was left to cool under cover.

The slide to be used was cleaned with ether and flamed on both sides taking special care to avoid bending of the glass.

In order to flame the coverslip after cleaning with ether, it was deposited on a cleaned, flamed slide and flamed while holding the two together with a forceps so as to prevent as much as possible any distortion of the coverslip.

As an alternative to these manipulations with glass slides and glass coverslips, a plastic slide and coverslip[1] were used. Here special care was taken not to touch them with the fingers and they were handled with ether-cleaned and flamed forceps.

Either a polyethylene pipette or a flamed glass pipette was used to withdraw a drop of blood suspension from the test-tube and to deposit it onto the slide.

All these precautions were found necessary in order to avoid crenation of the erythrocytes before beginning to decrease the salt concentration of the medium surrounding the cells.

Saline was prepared using fresh glass double-distilled water with buffer Michaelis (pH 7.4).

For the gradual reduction of the salt concentration, a mixed ion exchanger[2] was used. This mixed resin exchanges H for Na and OH for Cl ions from the medium surrounding the erythrocytes thus gradually reducing the salt concentration of the medium while maintaining, at the same time, a relatively constant pH. In general variation in pH was negligible, when it did occur, and was towards the acid side. The rate at which the Na and Cl ions are removed from the solution depends on the proportion of suspending medium to ion exchanger, on the one hand, and the area free from ion exchanger in the middle of the preparation, on the other. It was also found that embedding the ion exchanger powder in a very thin film of celluloid reduces the rate of ion exchange.

After taking into account these considerations, the method finally employed was the following: 0.5 gram of mixed ion exchanger was ground in a porcelain mortar, previously cleaned and rinsed with fresh glass double-distilled water and dried. The powder thus obtained was added to a test-tube containing a solution of 2% Parlodion[3] in doubly distilled amyl acetate. The suspension was allowed to sediment for 15 minutes. One-tenth milliliter of the supernatant was deposited on a cover glass. The drop spreads over a diameter of ca. 0.8 mm on the coverslip. After drying, the ion exchanger adheres to the glass or plastic coverslip. A central hole is now cut in the circular "film" in the shape of a rectangle with the help of a fragment of a razor blade. The size of the blade fragment used determines the dimensions of the rectangular hole in the center of the coverslip. The internal rectangle of embedded ion exchanger is peeled off. Two-tenths milliliter of the blood suspension is taken by the previously flamed glass pipette or by a clean polyethylene pipette and deposited on the slide. The slide is placed under the objective and the drop oriented above the light coming from the condenser. The drop is now covered with the treated coverslip so that the light coming from the condenser is directed to the center of the inner rectangle. The objective is focused and observation or cinematography is started. Both can be done at the same time using the Wild cinematography attachment. A Wild phase-contrast microscope was used and Cine-Kodak special II movie camera with Kodak +X negative film.

When electron microscopic examination is desired, the slide is dipped into a 0.3% Formvar solution in chloroform and dried under cover at room temperature. The gradual hemolysis is performed as previously described on the film covering the slide. When hemolysis was completed the other side of the slide is marked to designate the area of the rectangle within the ion exchanger. The coverslip is removed. If desired, fixation by vapors of OsO_4 or Formalin can be done at this stage. After drying, the slide is shadowcast. The grid is deposited on the film over the marked area and the film then floated on a water surface. After air drying the specimen is ready for observation.

RESULTS

The time necessary to obtain ghosts of a refractive index similar to the surrounding medium varied from less than one minute to 45 minutes according to the dimensions of the rectangle cut within

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[1] No. 7029A, Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.

[2] Amberlite MB-3, Rohm and Haas, Philadelphia, Pa., U.S.A.

[3] Mallinckrodt Chemical Works, New York, U.S.A.

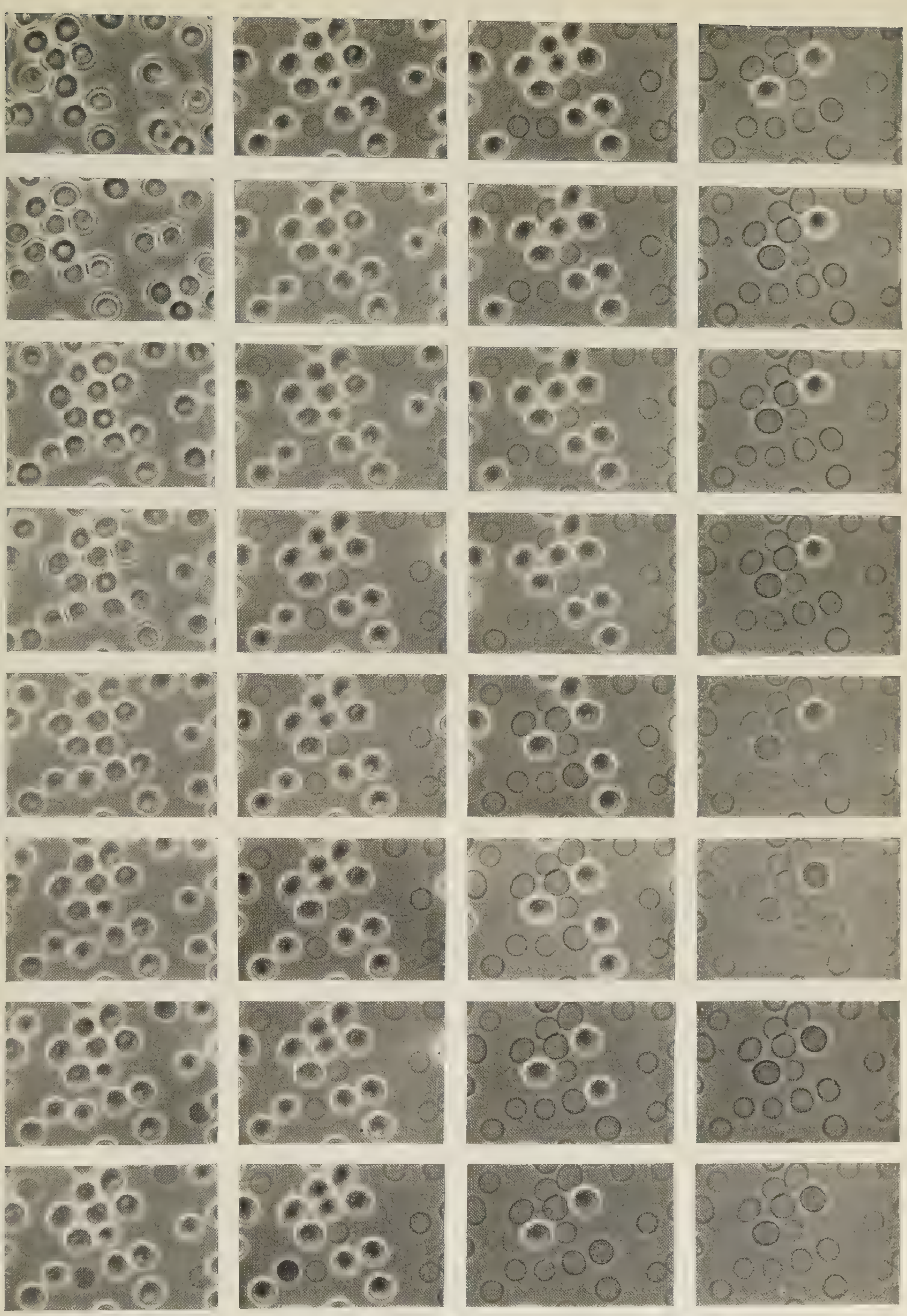

Fig. 1 Osmotic hemolysis by gradual decrease in the ionic strength of the surrounding medium. From a film sequence photographed at 16 frames per second under phase-contrast microscope. The photographic series begins at top left and continues down. There is a 30-second lapse between every two frames except for the last 4 frames which represent a lapse of only 5 seconds.

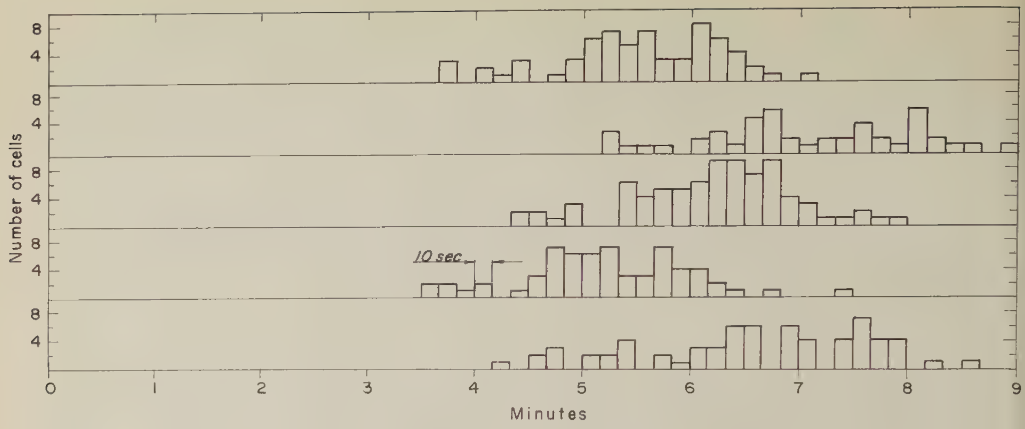

Fig. 2 A graph of 5 representative experiments showing the number of cells that hemolyzed per unit time (10 seconds) from the beginning of the experiment to total hemolysis (9 minutes). It should be emphasized that the measurement of time in this experimental system represents the ionic strength at a certain time and does not have any direct connection with the kinetics of hemolysis.

the mixed ion exchanger on the coverslip, the concentration of the resin and whether or not Parloidon embedding was used. In a given preparation the hemolysis was more rapid near the border of the ion exchanger and slower towards the center of the rectangle. Even apparently identical preparations showed variations in the time necessary for complete hemolysis (see fig. 2). When the hemolysis was very rapid (less than 40 seconds) some of the erythrocytes were seen to burst on one side and to be simultaneously thrown aside by the reaction force. In some instances a slight bulging of the membrane could be observed prior to the bursting followed by a discharge of optically dense solution thrown out of the cell at that point. When the hemolysis occurred slowly, the cell first became a sphere, and shortly afterwards the hemoglobin was released in all directions and the ghosts became increasingly pale until only the border was visible.

The sequence of deformation commenced from a bidiscoid form to a thicker bidiscoid form, of smaller diameter, then to a cap. The depression gradually became shallower and finally a sphere was observed. This sphere maintained its size till it suddenly slightly increased in diameter and released its contents. The cells in one field were never observed to burst at the same time no matter how quickly or slowly the hemolysis took place. There were always a few cells that released their hemoglobin very early and after the majority of the cells were transformed into ghosts, there were still several cells which retained their full spherical shape for a few seconds or even minutes. The sequence of these events is presented in figure 1; and figure 2 presents the number of cells releasing their hemoglobin as a function of time, in 5 representative experiments.

If the appropriate precautions were observed regarding cleanliness no crenation was observed during the process of osmotic hemolysis. A mere touch with the finger on one of the glasses was sufficient to produce crenation. Under the effect of the decreasing salt concentration the crenated cells gradually reversed to a bidiscoid form and then followed the sequence of events described in the preceding paragraph.

DISCUSSION

The sequence of the morphological changes followed under the microscope is in general agreement with the disc-sphere transformation for osmotic hemolysis as described by Ponder ('55), except for the rapid transition through the cap form as described by Ponder and which, in the present experiment, where hypotonicity was gradually produced, was noted to be a slower process. The rupture of the membrane with the discharge of hemoglobin as described by Comandon and Fonbrune ('29) though not confirmed by Bessis ('54) does sometimes occur in rapid hemolysis. An attempt was made to clarify the phenomenon of the bursting cells under the phase microscope by examining the preparation after lysis in the electron microscope. While most of the membranes present the appearance of the normal ghosts, somewhat shrunken and wrinkled due to the effect of drying in a hemoglobin-rich medium on the plastic film (fig. 3) in some of them a hole can be seen in the membrane (fig. 4). The observation of the slight increase in diam-

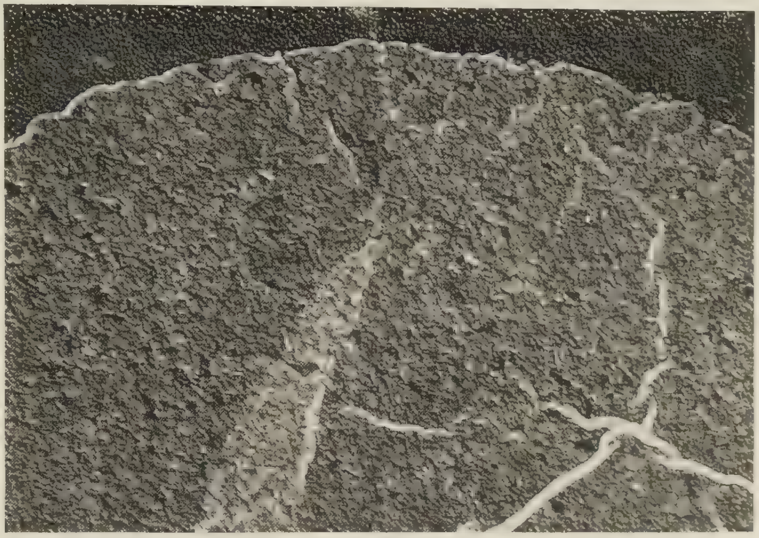

Fig. 3 Electron micrograph of red blood cell membrane obtained by slow hemolysis on a slide covered with a Formvar film. Hemoglobin was not washed out (note granularity of back-ground). Shadowcast by Pt 1:4. × 24,000.

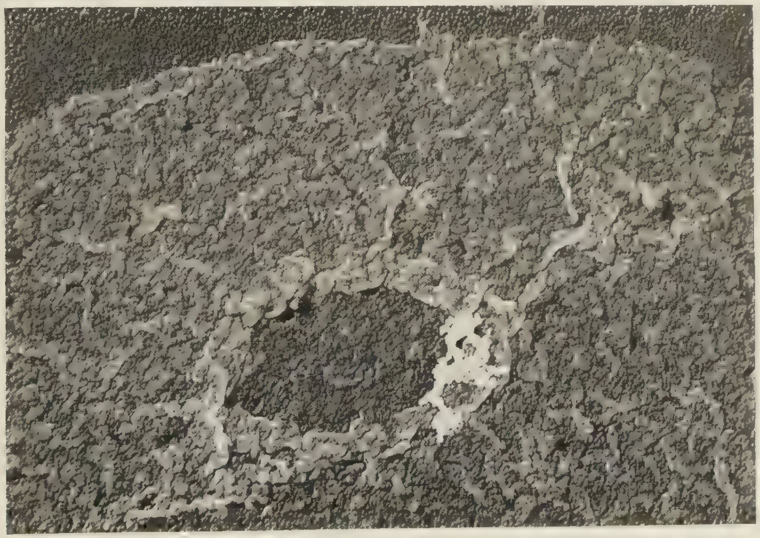

Fig. 4 Electron micrograph of a red blood cell membrane produced by rapid hemolysis using the same technique as for the preparation in figure 3. Note the hole in the membrane. × 24,000.

eter after the cell has become a sphere prior to the release of hemoglobin in gradual hemolysis is in agreement with the considerations of Katchalsky et al. ('61). These authors assume that a stress on the membrane starts after the volume of the cell cannot increase without increase of the surface area of the membrane. If an additional volume is added gradually, the membrane will augment in surface area (seen under the phase microscope as increase in the diameter of the sphere) and eventually open pores to let hemoglobin out. If this increase in volume is rapid, sufficient stress is developed, enough to break the membrane at one point, rather than stretching the membrane and opening pores over its entire surface. This phenomenon is assumed to be due to the visco-elastic behavior of the membrane while it is being stretched.

Three categories of cells may be classified according to the time that elapses from the beginning of the process described above to the point where the hemoglobin is discharged. The first group consists of those cells which undergo early lysis; the second group contains the majority of the cells and the third category consists of the cells which are late to lose their hemoglobin. It con be seen from figure 1, as well as from figure 2, that approximately 10% of the cells belong to the early lysing group (cells that release their hemoglobin earlier than the majority). The late lysing cells account for up to 4% of the total. All the cells, in the middle group follow one another closely in lysing. It can also be seen from figure 1 that the first cells to lose their hemoglobin are smaller in diameter as compared to those cells which are the last to lose their hemoglobin, and that they are also transformed to spheres at a more rapid rate. The data of Chalfin ('56), Hoffman ('58) and Marks ('58) indicate that the old cells are the first and the young cells the last to undergo hemolysis. The findings of Chalfin ('56) and Munn ('58) show that the cells decrease in volume as they mature. (See also Parpart, '51). Another confirmation of these findings is supplied by measuring the mean diameter of young and old cells with the help of a Coulter counter[4] after separation of the old from the young erythrocytes by the Allison ('55) or Prankerd ('58) methods. The mean diameter of the cells from the bottom layer (old cells) was found to be smaller than the mean diameter of the cells from the top layer (young cells). It may be concluded, therefore, that the small cells which were seen to undergo early hemolysis are old cells and the larger cells late to lose their hemoglobin, are young cells. The majority of the cells display a range of characteristics of an intermediate degree, being less fragile than the early to lyse cells (old cells) and less resistant than the late lysing (young cells). It is noteworthy that the percentage of early lysing cells is in agreement with the percentage of morphologically old cells obtained from normal donors as determined by electron microscopy of the red cell membranes (Danon and Marikovski, '61).

It is apparent that the method described above may be applied for the determination of the osmotic fragility of erythrocytes as well as to the study of the distribution of the erythrocytes into age groups. This would, of course, require a standard mixed ion exchanger film and a standard dilution of erythrocytes. The osmotic fragility might well be expressed in terms of time which elapses from the initial contact between the sample and the ion exchanger to the onset or completion of hemolysis.

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he Exchange of Radioactive Magnesium in rythrocytes of Several Species

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A previous publication (Rogers and lahan, '59) has described the exchange of dioactive magnesium in various tissues f the rat. It was found that there are two rincipal exchange rates, one with a turn- ver time of 1.2 hours and another with bout 25 hours. In myocardium, liver and idney, only the fast component was ap- arent and the specific activity of the tis- ue magnesium reached that of the plasma agnesium in three hours. In the other tis- ues studied (brain, skeletal muscle, testes nd red cells), more than half of the mag- esium exchanged at the slower rate. It as of interest to compare the exchange of dioactive magnesium in the red cells of ther species both *in vivo* and *in vitro*, and rat cells *in vitro*.

METHODS

For the *in vivo* studies, isotonic solu- ons of $MgCl_2$ containing Mg^{28} were in- cted i/v into dogs and cats. Blood sam- les were drawn at intervals from the atheterized femoral artery. In the *in vitro* udies, the radioactive solution was added whole blood of rats, dogs, cats, cattle nd man, which was then incubated at 8°C with gentle agitation under 5% car- on dioxide in oxygen. Samples were rawn at intervals.

In each case, the blood was immediately entrifuged at a standard rate for 4 min- tes and the plasma removed for radio- ctive and chemical assay. The cells were ashed with ice-cold isotonic saline, cen- ifuged for 4 minutes and then the wash- g and centrifugation were repeated once ore. An aliquot comprising 0.5 ml of ashed, packed red cells was added to 0.5 l water in a tube which was then placed a well-type scintillation counter. After ounting, 2 ml of 10% trichloracetic acid as added to the hemolysate and centrifuged in the same tube. The supernatant fluid was analyzed for magnesium by the method of Orange and Rhein ('51). Plasma samples received similar treatment. The results are expressed as the "relative specific activity" (r.s.a.) which is the ratio

$$\frac{\text{Specific activity of red cell Mg}}{\text{Specific activity of plasma Mg}}$$

When this ratio is equal to 1.0, equilibration is complete. An advantage of this method of expressing the data is that variations in the packing of the cells are unimportant because the count rate and magnesium content are determined for each 1 ml sample of hemolysate. In some instances the hemolysate was centrifuged and the supernatant counted (without the cell ghosts). In each case the count rate was identical with that in the original sample, indicating that the Mg^{28} content of the "membranes" was negligible.

RESULTS AND DISCUSSION

The *in vivo* and *in vitro* equilibration of the magnesium in cat red cells with that in the plasma is shown in figure 1; almost identical curves were obtained for dog and rat cells. The pattern of equilibration in rat erythrocytes *in vivo*, which was previously described (Rogers and Mahan, '59), was determined by sacrificing isotope-injected animals in series. All the results obtained *in vivo* tend to lie on less smooth curves than those *in vitro* and this may be partly attributed to the rapid biological decay of the plasma magnesium radioactivity and the consequently greater likelihood of error in its determination. Nevertheless, the similarity of the *in vivo* and *in vitro* magnesium exchange is quite clear. These results also show that in the cat and the dog, the exchange of red cell magnesium with that of the plasma is similar to that reported for the rat. The "fast" component accounts for 20–30% of the total magnesium.

The *in vitro* equilibration in human red cells is shown in figure 2. It will be noted that the scale of the abscissa in this graph is 10 times that in figure 1. The equilibration in red cells from these two human donors is much slower than in the othe species; it was so slow that the count-rat was too low for optimal accuracy in th earlier samples. In the bovine red cell the exchange was even slower and no rel able count-rate could be measured fo them at any stage of the incubation.

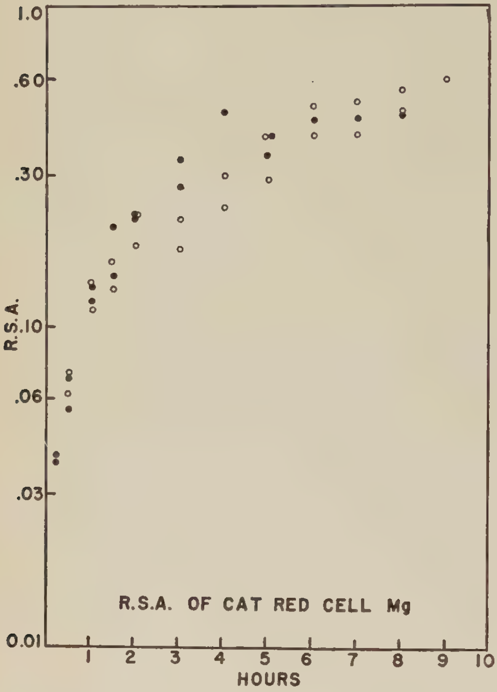

Fig. 1 Open circles represent *in vitro* data; solid disks *in vivo*.

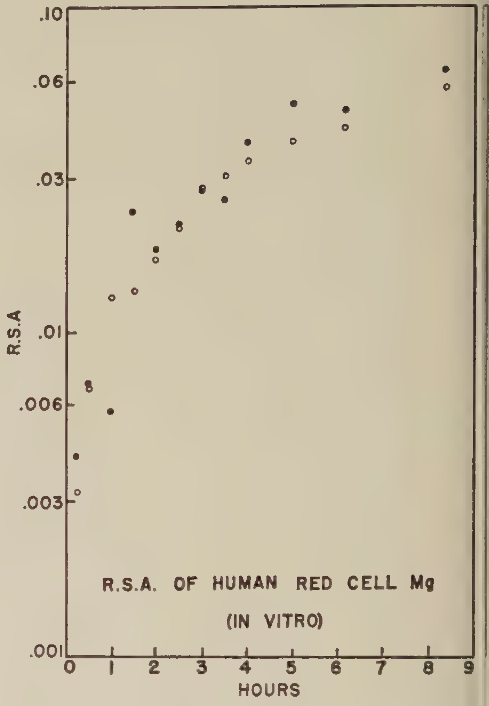

Fig. 2 Open circles represent *in vitro* data solid disks *in vivo*.

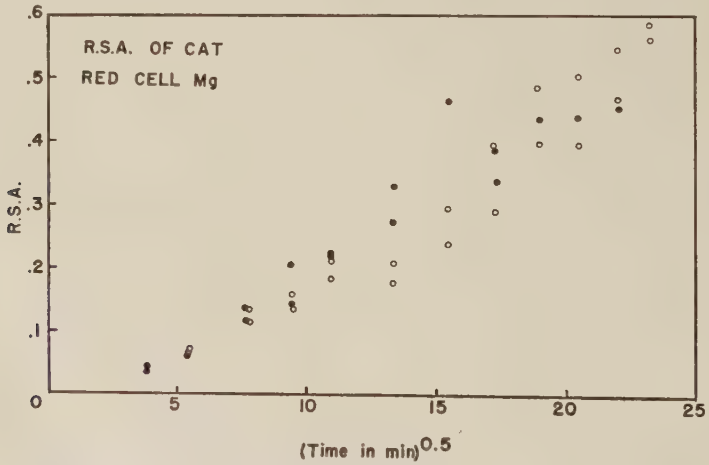

Fig. 3 Open circles represent *in vitro* data; solid disks *in vivo*.

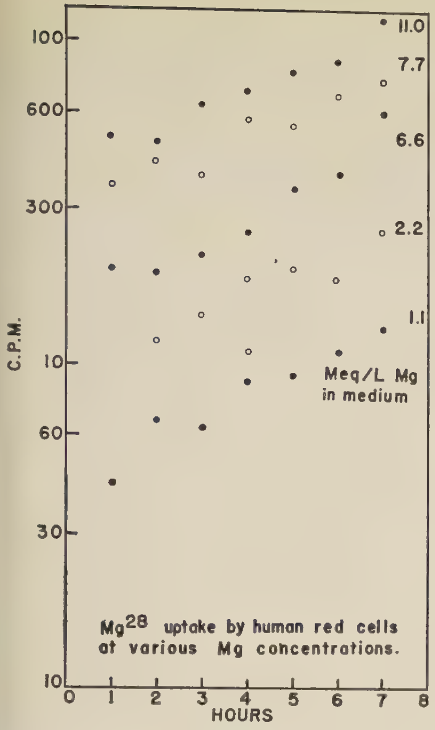

Fig. 4 Open and solid circles are used alterately to distinguish between the data relating each magnesium concentration.

Red cells, therefore, not only contain ss magnesium than other tissues (5.0 g/100 ml cells as compared with 18.0 g/100 gm muscle, in man), but even is small content is very slowly exchangeble.

Harris and Prankerd ('57) have shown at the permeation of potassium in the ythrocytes of man and the dog has the haracteristics of the diffusion of particles to a flat sheet with a resistive surface. he critical characteristic is the linearity f a considerable portion of the plot of acer K uptake against the square root of me. The mathematical rationale of this near relationship is given in the appendix to their paper. In figure 3, the *in vivo* and *in vitro* equilibration of cat red cell magnesium is plotted against the square root of time. Similar plots were obtained for the other species, both *in vivo* and *in vitro,* all suggesting that the postulate of Harris and Prankerd is also applicable to the permeation of erythrocyte magnesium.

Because of the low specific activity of the Mg^{28} presently available, it is impractical to achieve a purely tracer dose of this isotope; a small magnesium load is inevitable in nearly all these experiments. A possibility to be considered therefore, is that the "fast" component of the uptake of isotope by the cells may be in part accounted for by adsorption in superficial portions of the cells, and not strictly by exchange of Mg^{28} for Mg^{24}. Accordingly, a series of Krebs-Ringer bicarbonate-CO_2 buffered solutions was prepared, with varying magnesium concentration but with the same specific activity of the magnesium. Human red cells were incubated in these solutions and their uptake of radioactive magnesium is plotted in figure 4. The extent of the initial "fast" uptake of isotope is clearly dependent on the concentration, but the "slow" component seems unaffected by it. A suitable index of the extent of the initial uptake of isotope is the intercept value given by extrapolating the "slow" equilibration lines back to the abscissa. These values bear a linear relationship to the magnesium concentration in the incubating medium.

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A Comparative Study of the Effect of Ryanodine on Muscle[1,2]

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Previous investigations of the pharmaology of ryanodine have demonstrated hat crab (Edwards, '47) and insect muscle (Edwards, '48) are sensitive to this gent, in addition to that of all vertebrates tudied (e.g., Procita, '56 and Blum et al., 57). The report of mitotic inhibition by yanodine (Cornman, '51) suggested that he drug might have a more general inuence on contractile systems. However, motility was shown to be unaffected in *Proteus morgani* and *Trichomonas vaginalis,* and early experiments in this laboraory indicated that some invertebrates were also unaffected. In view of the negative nature of much of the evidence pertaining to the mechanism of action of yanodine (Blum, '57), a comparative survey of sensitivity to this drug was underaken, with the object of correlating it with known biochemical and morphologcal characteristics of each muscle and species, and providing thereby a basis for a more direct approach. A preliminary account of some of this work has been published (Haslett and Jenden, '59 and enden and Haslett, '60).

METHODS

Isolated tissue preparations were employed in assessing the effect of ryanodine n most of the species studied. Strips of muscle, thin enough to allow for adequate diffusion of drugs and oxygen, were mounted on electrodes and suspended in physiological saline solutions. The media used for suspending the muscles are deailed in table 1. Krebs bicarbonate soluion was used for chick and human muscle. In experiments with invertebrate, hark and alligator muscle, temperature was controlled by conducting the experiments in a room at 17 to 18°C; mammalian and avian muscle was examined at 38°C. The behavior of the muscles was recorded either kymographically with an isotonic lever, or isometrically with a strain gauge and strip chart recorder.

Maximal tetanic stimulation was delivered from Grass square wave stimulators through platinum mass electrodes to determine the maximal contraction of which each muscle was capable and to produce regular intermittent activity in the tissue. Trains of stimuli were delivered at intervals of approximately 100 seconds; the duration and frequency were adjusted individually for each muscle to produce a maximum effect.

Dissection of muscle preparations

Strips of longitudinal body wall muscle were used in the case of *Nereis virens, Lumbrenereis, Lumbricus terrestris, Thelepus crispus, Stichopus californicus,* and *Caudina arenicola.* A strip of one of the ventral retractors was taken from *Sipunculus nudus* by separating a thin bundle of fibers from the main band with a blunt dissecting needle. Three different preparations were used from *Octopus bimaculatus:* from the mantle, the lateral surface of the tentacle and the gizzard. In each case a strip of muscle about 5 mm wide and 2 cm in length was used. The pedal retractor was found to be the most

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[1] The investigation described in this paper was supported by research grant B-1663 from the United States Public Health Service.

[2] The authors acknowledge with gratitude the helpful advice of Dr. B. C. Abbott and Dr. R. A. Boolootian, and the technical assistance of Mr. D. E. Zinn in part of this work.

[3] Most of the work herein described was included in a thesis submitted by one of us (W. L. H.) in partial fulfillment of the requirements for the Master of Science degree at the University of California, Los Angeles.

TABLE 1

Composition of solutions used for isolated muscle preparations

Species	Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	Br^-	$SO_4^=$	HCO_3^-	$HPO_4^= + H_2PO_4^-$	Urea	Reference
Lumbrenereis *Thelepus crispus* *Nereis virens* *Sipunculus nudus* *Octopus bimaculatus* *Mytilus californianus* *Strongylocentrotus franciscanus* *Stichopus californicus* *Caudina arenicola*	458.6	9.8	10.1	52.7	535.4	0.5	27.8	2.5	0	0	Pantin, '48
Lumbricus terrestris	135.9	2.7	1.8	0.4	142	0	0.4	0	1.0[1]	0	Pantin, '48
Panulirus interruptis	500.2	14.0	14.4	6.8	540.9	0	7.9	0	0	0	Pantin, '48
Loxhorhynchus grandis	494.1	11.3	12.6	18.4	536.5	0	15.4	0	0	0	Pantin, '48
Romalea microptera	140.0	10.0	2.0	2.0	148.0	0	0	4.0	6.0	0	Hoyle, '53
Alligator mississippiensis	122.4	1.9	2.2	0	126.3	0	0	2.4	0	0	Clark, '57
Heterodontus francisci	277.9	11.3	4.7	6.8	304.0	0	4.0	0	0	394.2	Pantin, '48

[1] Solution adjusted to pH 7.4 by addition of hydrochloric acid.

uitable muscle from *Mytilus californi-nus;* it was removed together with the edal, which provided a convenient means f attaching the thread at one end. The nterior byssus retractor was also used in ome experiments. One of the lantern rotractor muscles was removed from the ea urchin, *Strongyloncentrotus francis-anus,* along with its origin on the aboral nd of the alveolus and insertion in the nterambulacral area. Attachments were made to the origin and insertion of the muscle, thus avoiding damage to the mus-le itself.

Small groups of muscle fibers were aken from the meropodites of the first, econd and third pereiopods of both *Loxorhynchus grandis* and *Panulirus interruptus,* attached at one end to a small egment of apodeme and at the other, to piece of the exoskeleton. The flexor ibialis muscle was isolated from the emur of the lubber grasshopper, *Romalea microptera.* The apodeme and coxa provided convenient attachments. The isolated semimembranosus muscle of a 4-day-old chick provided a satisfactory preparation of avian muscle. From the orn shark, *Heterodontus francisci,* strips f ventral constrictor muscle were isolated rom the lower jaw. Reptilian muscle was epresented by a strip of flexor muscle rom the thigh of *Alligator mississippien-is.*

Isolated preparations of frog rectus abdominis and sartorius, and of mammalian ntestine, uterus, bladder and diaphragm were dissected out and studied by conventional techniques. Segments of intestine examined included duodenum, je-unum and ileum.

Microorganisms

The effect of ryanodine on bacterial growth was assessed by the agar cup method described by Erlanson ('51), and y following the turbidity of broth cultures olorimetrically. Effects on bacterial motility were assessed by two methods; in he first, the organisms were incubated in roth containing ryanodine and then treaked along one edge of a plate. The ate of swarming was compared with that hown by a control culture. In the second method, centrally located agar cups were filled with preincubated mixtures of broth, ryanodine and bacteria, and the rates of spread peripherally were compared with controls.

Protozoa were examined visually with a Baker interference microscope. In the case of *Amoeba proteus,* ryanodine uptake was insured by inducing pinocytosis (Chapman-Andreson, '56).

Human semen was allowed to stand at room temperature in the presence of 10^{-4} gm/ml ryanodine. The percentage of motile sperm was determined microscopically and compared with a control in the absence of ryanodine at intervals up to 23 hours.

Identification of phosphagens

Samples of muscle from most of the species examined were subjected to the first stage of the extraction procedure of Ennor, Morrison and Rosenberg ('56) for the isolation of phosphagens. The crude barium salts were hydrolyzed by dissolving in 1 N HCl and allowed to stand at room temperature for 24 hours. The hydrolyzed extracts were spotted on Whatman 3 MM paper with arginine, creatine, glycocyamine and taurocyamine as marker compounds and developed at 25°C by descending chromatography. Two solvents, butanol-6 N HCl(70:30, v/v) (Makisumi, '53) and phenol water (80:20, w/v) saturated with SO_2 (Crawford, '51), were used and gave excellent separation of these guanidines. Sufficient extract was applied to each spot to enable detection of either creatine or arginine by means of the diacetyl test described by Anderson and coworkers ('57). Duplicate chromatograms were run and subjected to the reaction described by Jepson and Smith ('53) for arginine and other guanidines.

Determination of phosphagen content

For quantitative estimation of phosphagens, the muscle preparation was dissected out and suspended in oxygenated sea water for several minutes to allow recovery from the trauma of dissection. It was then plunged into freezing isopentane (− 160°C) for a minute or two, weighed, and powdered by a sharp blow. The powder was homogenized in a Sorvall

TABLE 2

Summary of responses of isolated preparations to ryanodine, and phosphagens found in each species

Phylum and species	Phosphagen[1]	Muscle	Response to ryanodine
Proteus morgani	?	—	None
Escherichia coli	?	—	None
Lactobacillus arubinosus	?	—	None
Staphylococcus aurens	?	—	None
Protozoa			
Amoeba proteus	?	—	None
Tetrahymena geleii	?	—	None
Stentor sp.	?	—	None
Spirostomum ambiguum	?	—	None
Annelida			
Lumbricus terrestris	LP (Thoai, '54)	Body wall	?
Lumbrenereis sp.	CP	Body wall	?
Thelepus crispus	AP	Body wall	None
Nereis virens	GP + CP	Body wall	Contractile failure
Sipunculoidea			
Sipunculus nudus	AP	Ventral retractor	None
Mollusca			
Octopus bimaculatus	AP	Mantle	None
		Tentacle	None
		Gizzard	None
Mytilus californianus	AP	Pedal retractor	None
		Anterior byssus retractor	None
Holothuroidea			
Strongylocentrotus franciscanus	CP	Lantern protractor	None
Stichopus californicus	AP	Longitudinal muscle	Contractile failure
Caudina arenicola	AP	Longitudinal muscle	Contractile failure
Arthropoda			
Panulirus interruptis	AP	Carpopodite adductor	Rigor
Loxhorrhynchus grandis	AP	Carpopodite adductor	Rigor
Romalea microptera	AP[2]	Flexor tibialis	Contractile failure
Chordata			
Heterodontus francisci	CP	Ventral constrictor of lower jaw	Rigor
Alligator mississippiensis	CP	Biceps brachialis	Rigor
Rana pipiens	CP	Sartorius	Rigor
Four-day old chick	CP	Rectus abdominus	Rigor
		Semimembranosus	Rigor
Rat	CP	Diaphragm	Rigor
		Intestine	Rigor
		Uterus	None
Rabbit	CP	Intestine	Rigor
		Uterus	None
		Bladder	None
Guinea pig	CP	Diaphragm	Rigor
		Intestine	Rigor
		Uterus	None
Man	CP	Intercostal	Rigor
		Sperm (motility)	None

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[1] CP, creatine phosphate; AP, arginine phosphate; GP, glycocyamine phosphate; LP, lombricin phosphate.

[2] The phosphagen of this species has not been examined, but analysis of many related species suggests that only AP is present.

omnimixer in about 50 volumes of 70% v/v ethanol below 0°C and the homogenate was centrifuged. To the clear supernatent was added 1.0 M barium acetate until no further precipitate was obtained, and the pH was adjusted to 9.0. After standing overnight at − 16°, the suspension was centrifuged and the ppt dissolved in 0.1 N HCl at 100°C for 10 minutes. The resulting solution was made up to 25, 50 or 100 ml and aliquots were subjected to guanidine estimation by the modified diacetyl method described by Rosenberg et al., ('56). *Nereis virens* has been shown to contain both creatine and glycocyamine phosphates; simultaneous estimation of both guanidines was achieved by running the diacetyl test both in the absence and in the presence of n-propanol, which was shown to increase the color produced by glycocyamine but decrease the creatine color intensity. The concentrations of both bases were estimated by comparison with standard curves, which indicated summation of the color intensity in the presence of a mixture.

Creatine and arginine phosphokinase

Creatine phosphokinase was prepared from rabbit muscle by the method of Kuby, Noda and Lardy ('54) and estimated both by the procedure described by these workers and by a titrimetric method (Cho, Haslett and Jenden, '60). Arginine phosphokinase was prepared from the tail muscles of the spiny lobster, *Panuliris interruptis* and estimated by the methods described by Morrison, Griffiths and Ennor ('57). The influence of ryanodine on these enzymes was assessed by including the alkaloid in the reaction mixture in concentrations up to 10^{-4} M, and by preincubating each enzyme in the absence of substrate with ryanodine in concentrations up to 3×10^{-3} M.

RESULTS

Table 2 presents a summary of the responses to ryanodine which were observed in each species. No changes in growth rate or motility were observed in any of the bacteria studied at ryanodine concentrations of up to 10^{-3} gm/ml. Similarly the motility and behavior of all 4 of the protozoa studied were unchanged after 1 to 5 hours exposure to ryanodine at concentrations of $3\text{–}5 \times 10^{-4}$ gm/ml. Human sperm were also unaffected at 10^{-4} gm/ml after 23 hours.

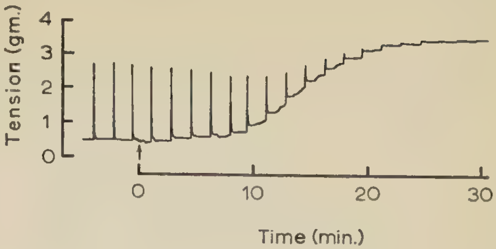

Fig. 1 Isometric recording showing the influence of ryanodine (2.5×10^{-5} gm/ml) on the flexor muscle of *Panulirus interruptis*. Ryanodine was added at the arrow. Tetanic stimuli of three seconds duration were applied every 100 seconds. Pulse duration 5 msec; frequency 100/sec. For further details see text.

Three types of result were obtained on exposure of isolated muscles to ryanodine. In some muscles an irreversible contracture was seen (fig. 1), similar to that previously described in vertebrate skeletal muscle. Other muscles showed a progressive decline in both spontaneous and induced contractions (fig. 2), similar to that seen in mammalian cardiac muscle (Furchgott and de Gubareff, '56). Muscle from several species appeared to be completely unaffected by the drug (fig. 3). A contracture was seen in crustacean muscle, vertebrate skeletal muscle, and most of the vertebrate smooth muscles studied. Its characteristics were very similar from species to species and no significant differences were seen.[4] It was markedly accelerated by spontaneous or induced contractions, and during its later stages was associated with a failure of the mechanical response to stimulation. In all cases the effect was irreversible, although as in the case of frog rectus abdominis (Blum et al., '57) a decay in isometric

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[4] The relative tension developed in response to ryanodine and to maximal tetanic stimulation varied a good deal among different muscles, and was frequently greater following ryanodine. The variation was correlated with the thickness of the muscle, and is probably attributable to differences in the degree of synchronization of response to ryanodine in different parts of the muscle preparation, since the tension decays after the peak is achieved isometrically (Blum et al., '57).

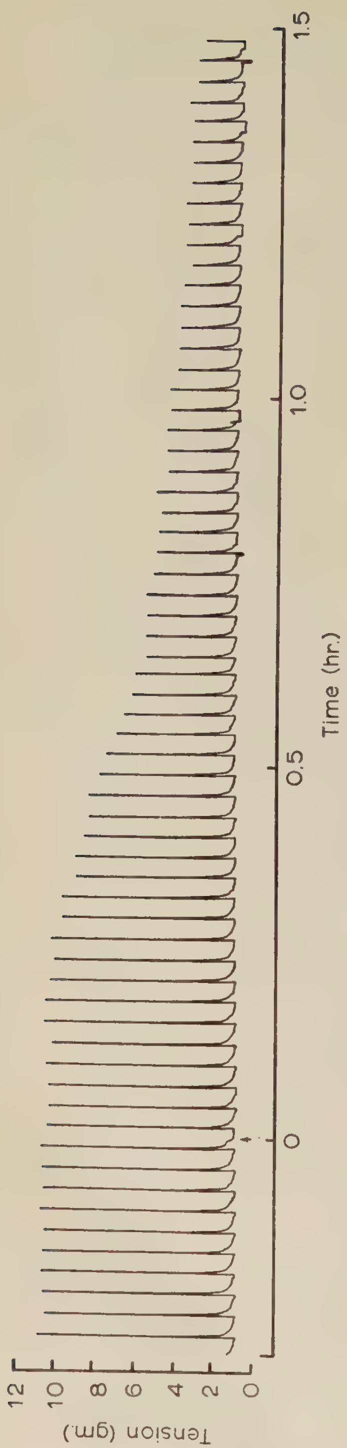

Fig. 2 Isometric recording showing the effect of ryanodine (2.5×10^{-5} gm/ml) on body wall muscle of *Nereis virens*. Ryanodine was added at the arrow. Tetanic stimuli of two seconds duration were applied every 100 seconds. Pulse duration 5 msec; frequency 20/sec. The muscle was suspended in oxygenated, artificial sea water at 17°C.

tension was observed following the peak effect and appeared to be associated with tearing of the muscle fibers.

A progressive contractile failure was seen in muscle from *Nereis virens, Stichopus californicus, Caudina arenicola,* and *Romalea microptera.* Consistent responses to electrical stimulation could not be obtained in Lumbrenereis and Lumbricus terrestris, and the spontaneous rhythmic activity of muscle from these animals was too irregular to allow an accurate assessment of the effects of ryanodine. There was some suggestion of a reduction in spontaneous activity in Lumbricus terrestris. The effect of iodoacetate (1.0 mM) on muscle from *Nereis virens* and *Romalea microptera* was examined in order to determine the ability of the muscle to undergo a sustained contracture. Although both a contracture and a progressive failure of contractility was seen in the insect muscle, the tone and response to electrical stimulation in *Nereis virens* were unaffected after more than 1.5 hours. Barium chloride (4×10^{-5} gm/ml) also produced no observable effect after 1.5 hours, and 2,4-dinitrophenol (5×10^{-5} M) had no visible effect over a three-hour period; the muscle was still capable of responding to electrical stimulation at the end of 15 hours exposure to this compound. Although Needham and coworkers ('32) claimed to have produced heat rigor in *Nereis diversicolor,* prolonged exposure to heat was ineffective in inducing a contracture in isolated muscle from *Nereis virens.*

There was some evidence that the last stage of relaxation after tetanic stimulation was slower in the muscle preparation from *Romalea microptera* in the presence of ryanodine than in its absence; this phenomenon is being studied further and will be described in detail in a subsequent report.

No differences in tone or in contractility between control muscles and muscles exposed to ryanodine (2.5×10^{-5} gm/ml) were seen in *Thelepus crispus, Sipunculus nudus, Octopus bimaculatus, Mytilis californianus,* and *Strongylocentrotus franciscanus;* in addition, isolated rat, rabbit and guinea pig uterus and rabbit bladder preparations were apparently unaffected.

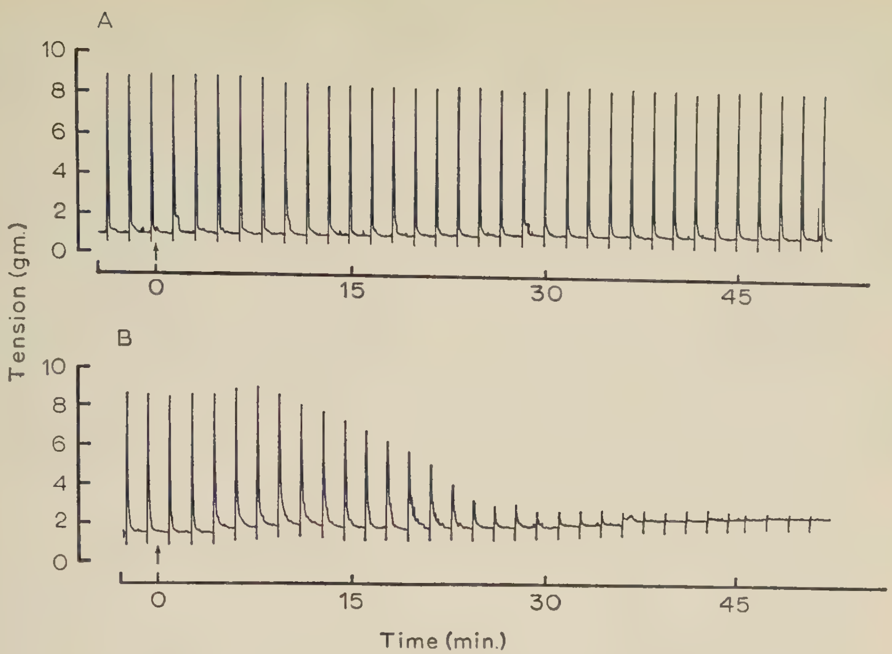

Fig. 3 Isometric recordings showing the effect of ryanodine (2.5×10^{-5} gm/ml; record A) and iodoacetic acid (1.0 mM; record B) on isolated strips of tentacle muscle from *Octopus bimaculatus*. Each drug was added at the arrow. Tetanic stimuli of three seconds duration were applied every 100 seconds. Pulse duration 5 msec; frequency 40/sec. The muscles were suspended in oxygenated, artificial sea water at 18°C.

Chromatographic studies

Although the phosphagens occurring in each class of animal used in the present study have previously been identified, the particular species used has generally not been studied, and chromatographic identification of the guanidine phosphates occurring in tissue extracts from each of the species examined was therefore carried out. The results are summarized in table 3. In all cases the unknown compounds yielded R_f values that were identifiable as creatine, arginine, glycocyamine or taurocyamine.

In view of the possible importance of phosphagen levels as a correlate of ryanodine sensitivity (see discussion), quantitative estimations of phosphagen content were made on certain species. In every case the phosphagen levels were found to be lower than previous estimates made in related species; these were based upon acid-hydrolyzable phosphate, which includes other components than phosphagen (Ennor and Rosenberg, '52 and Ennor and Morrison, '58). The results are summarized in table 4.

Enzymatic studies

Arginine phosphokinase was completely unaffected by ryanodine under all conditions and at all concentrations tested. Similarly, no inhibition of creatine phosphokinase was seen when ryanodine, 10^{-4} M, was included in the reaction mixture. Preincubation with ryanodine in the absence of substrate caused some inactivation, as Hill et al., ('58) have previously shown; however, this could only be demonstrated at concentrations of 3×10^{-4} M or above and at 40°C.

DISCUSSION

Cornman ('51) has described inhibition of mitosis by ryanodine in Echinarachnius eggs and embryonic mouse heart in tissue culture, and Bodenstein ('50) showed that the development of *Amblystoma punctatum* embryos could be retarded by ryanodine. These observations suggest that the alkaloid may affect some step which is

TABLE 3

The phosphagen content of the musculature of certain invertebrates as determined by the paper chromatography of acid-hydrolyzed barium salts

Tissue extract or marker substance	R_f values in	
	Butanol 6 N HCl	Phenol water SO_2
Creatine	0.42	0.70
Arginine	0.17	0.36
Glycocyamine	trails	0.53
Taurocyamine	0.36	0.78
Lumbrenereis sp.	0.41	0.70
Nereis virens	0.43; trails	0.71; 0.54
Thelepus crispus	0.17	0.37
Sipunculus nudus	0.17	0.36
Octopus bimaculatus	0.18	0.37
Mytilus californianus	0.17	0.37
Loxhorhynchus grandis	0.17	0.35
Panulirus interruptis	0.17	0.35
Strongylocentrotus franciscanus	0.41	0.71
Stichopus californicus	0.18	0.36
Caudina arenicola	0.18	0.36
Heterodontus francisci	0.41	0.70
Other vertebrates	0.42	0.70

TABLE 4

Phosphagen levels in some invertebrates (figures are mean ± standard error)

Species	Preparation	Number of experiments	Phosphagen	Phosphagen content (μmol/gm wet wt.)
Sipunculus nudus	Ventral retractor	3	AP	9.6 ±1.5
Nereis virens	Whole organism	3	CP	0.70±0.06
			GP	6.7 ±0.2
Octopus bimaculatus	Mantle muscle	3	AP	3.3 ±0.4
	Tentacle muscle	3	AP	1.7 ±0.4

essential in the normal activity of contractile systems other than muscle, and in particular contraction of the mitotic spindle. For this reason the present study included observations on the growth and motility of microorganisms and the motility of protozoa and of sperm. The fact that no positive effect of the drug was seen on any of these, including ciliary, flagellate and ameboid movement, makes it unlikely that the mechanism affected is a universal component of contractile systems. In both of the studies referred to above, the ryanodine concentration used was high (6–8 mg/ml in Cornman's work). Moreover a crude aqueous extract was used by Bodenstein ('50) rather than pure ryanodine;[5] such an extract undoubtedly contains a variety of pharmacologically active principles. In view of these facts it is possible that the effects on mitosis seen in these experiments are unrelated to the specific and extremely powerful actions of the drug on muscle. In the present study a positive action of ryanodine was seen only in muscle.

Two kinds of positive response of muscle to ryanodine have been observed: a progressive contractile failure and rigor. In addition, many muscles were apparently unaffected by the drug. These grades of sensitivity are all represented in mammalian muscle; cardiac muscle undergoes progressive contractile failure (Hillyard and Procita, '59) skeletal muscle contracts irreversibly and isolated strips of uterus and bladder retain their normal physiological properties. The flexor tibiae of the grasshopper was anomalous, inasmuch as some inhibition of relaxation was evident although the predominant response was contractile failure, and no contracture was observed. Mammalian intestinal strips have previously been shown to respond in a characteristic manner (Hillyard and Procita, '58); in this case, the basal tone is permanently increased by ryanodine, but relaxation may be induced by epinephrine or antispasmodics for as long as they remain in contact with the muscle.

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[5] Cornman ('51) gives no information as to the source or purity of the ryanodine he used.

No morphological or physiological characteristic has been discovered which permits an accurate prediction to be made of the particular type of response which a given species or muscle will show to ryanodine. In particular, the presence of striations, the size and microscopic features of the muscle cells, the speed and force of contraction and ability to maintain tension do not show any necessary correlation with the observed response to the drug. There is nevertheless a general correlation between sensitivity and the degree of phylogenetic development. Most of the insensitive species are at the lower end of the evolutionary scale, while only vertebrates and crustaceans showed rigor; a number of species of intermediate development showed contractile failure.

Special attention was paid to the identification of the phosphagen contained in each muscle studied. Its chemical identity is known to vary from species to species (Ennor and Morrison, '58) and in mammalian muscle at least, the creatine phosphate-phosphokinase system is capable of exerting a profound influence on the ability of glycerol-extracted muscle fibers to relax (Goodall and Szent-Györgyi, '53 and Lorand, '53). Since previous work on ryanodine led to the suggestion that its primary effect might be on relaxation, it seemed likely that sensitivity might be related to the phosphagen used. Tables 2 and 3 show that no obvious relation exists between the identity of the phosphagen and sensitivity; rigor occurred not only in vertebrates, but also in crustacea, which contain arginine phosphate in place of creatine phosphate. Contractile failure also occurs in animals containing either phosphagen, and one species containing only creatine phosphate (*Strongylocentrotus franciscanus*) showed no positive response to ryanodine. Finally, even in mammals, not all muscle is sensitive to the drug; uterine muscle appears to be completely unaffected, even when repeatedly tetanized.

There is some indication that the normal phosphagen levels in a particular type of muscle are correlated with the response of the muscle to ryanodine. Striated muscle which responds to ryanodine with rigor has in common a phosphagen level in the range of 20–30 μmol/gm wet weight, according to the best and most recent estimates[6] (Ennor, Morrison and Rosenberg, '56; Bendall and Davey, '57 and Carlson and Siger, '60); on the other hand, those types of muscle which have been found to be insensitive to ryanodine or to respond with contractile failure contain very much less. For example, in mammals, cardiac muscle contains 3–4 μmol/gm (Ennor and Rosenberg, '52; Furchgott and de Gubareff, '58), and uterine muscle has only about 1 μmol/gm (Menkes and Csapo, '52; Alekseeva and Kok, '52). Insect muscle has also been reported to contain very little phosphagen (Baldwin and Needham, '33–'34; Heller, '36), and although the estimation procedures used were not so refined as those now available, a more recent report indicates that the arginine phosphate concentration is only a fraction of that of ATP (Winteringham, '60). The figures for invertebrate smooth muscle, in which ryanodine rigor was never observed, are meager (Hanson and Lowy, '60) and most of these are open to the criticism that acid-hydrolyzable phosphate rather than phosphagen was determined. This component may include other phosphate esters, and is therefore subject to a potentially large positive error (Ennor and Rosenberg, '52; Ennor and Morrison, '58). The values obtained in the present work reflect only the guanidine phosphates contained in each species, and are somewhat lower than previous mean values reported for related species (Needham et al., '32; Baldwin and Yudkin, '50; Hanson and Lowy, '60). Mammalian intestine appears to represent an exception to the correlation drawn above, since the creatine phosphate level is only 3–4 μmol/gm, (Kroeger and Edwards, '53), yet ryanodine induces contracture. However, it has already been noted that the response of intestinal strips to ryanodine differs fundamentally from that seen in skeletal muscle in that the muscle will subsequently relax on exposure to antispasmodics (Hillyard and Procita, '58).

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[6] Some of the values reported for frog muscle extend somewhat below this range.

The correlation between high phosphagen levels and the induction of rigor by ryanodine, although tenuous, suggests that there may be in these muscles a specific phosphagen-dependent mechanism which is either obstructed by ryanodine or necessary for this agent to produce an effect. The generally accepted ATP-restoring function does not appear to be involved, in spite of the report of Hill et al., ('58) since the ryanodine concentration necessary to inhibit creatine phosphokinase is in our experiments about 1000 times that capable of causing a contracture at the same temperature; moreover, arginine phosphokinase was completely unaffected even though crustacean muscle shows a response similar to that seen in vertebrate skeletal muscle. It is possible that in these muscles phosphagen is directly involved in some process other than ATP restoration, with which ryanodine interferes; alternatively, the occurrence of high phosphagen levels in muscles responding to ryanodine with contracture may reflect the development of a ryanodine-sensitive mechanism with a critical ATP requirement.

The data reported here give no indication as to the significance of contractile failure as an alternative response to ryanodine. The very high potency of the compound suggests specificity of action; if the same basic biochemical lesion is produced irrespective of species and muscle type, then we must conclude that the process affected by ryanodine is coupled differently in different muscles, and that phosphagen may play a supporting, rather than a direct, role in this process.

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